Remarks

Reconsideration and allowance of the present application in view of the amendments above and comments which follow are respectfully requested.

In the Office Action dated September 30, 2004, the Examiner stated that claims 1-9, 15-27, 33-45, and 51-54 are elected in view of Applicant's election without traverse of species A directed to bacteria, and that claims 10-14, 28-32, and 46-50 are withdrawn from further consideration pursuant to 37 CFR 1.142(b) as being drawn to a nonelected species.

The Examiner objected to the specification because on page 5 at line 7, the word "identification" is misspelled. Applicant is correcting this by amendment.

The Examiner rejected claims 1-9, 15-27, 33-45, and 51-54 as being allegedly indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

The Examiner stated that the term "maximally" in claims 1, 19, and 37 is a relative term which renders the claim indefinite. The Examiner stated that term "maximally" is not defined by the claim, the specification does not provide a standard for ascertaining the requisite degree, and one of ordinary skill in the art would not be reasonably apprised of the scope of the invention.

Without agreeing to the correctness of the Examiner's position, but solely to advance prosecution, applicant has amended these claims to delete the term "maximally".

The Examiner rejected claims 1, 6-9, 17, 37, 42-45, and 53 as being allegedly anticipated by Himmelreich et al (MAGMA (2000) Vol. 11, Suppl. 1, page 199). The Examiner stated that Himmelreich et al. teach a MR spectroscopy method to identify metabolites that distinguish between microorganisms and classify them by multivariant analysis. The Examiner stated that, in regard to claims 1 and 37, Himmelreich et al. teach obtaining magnetic resonance spectra

(paragraph 1), decomposing spectra into integral regions (paragraph 2), and classifying using cross-validation and linear discriminant analysis (paragraph 3). The Examiner stated that, in regard to claims 6-9 and 42-45, Himmelrich et al. identify 28 species of bacteria, including Staphylococcus, Enterococcus, and Streptococcus species (paragraph 2). The Examiner stated that, in regard to claims 17 and 53, the specimens were from cultured bacteria (paragraph 2).

In response to this rejection applicant notes that the publication date of Himmelrich et al. was not before September 14, 2000. The listed authors are the same five persons who are the named inventors herein. The present application claims priority on U.S. provisional application serial no. 60/270,367, filed February 21, 2001. Because the Himmelrich et al. reference was not published more than one year before the priority date of February 21, 2001, applicant requests withdrawal of this rejection based on 35 U.S.C. §102(b).

The Examiner rejected Claims 1-9, 15-27, 33-45, and 51-54 as being allegedly obvious over Somorjai et al. (Magnetic Resonance in Medicine (1995) Vol. 33, pages 257-263; PTO Form 1449 Reference 21), in view of Delpassand et al. (Journal of Clinical Microbiology (1995) May, page 1258-1262; PTO Form 1449 Reference 5).

The Examiner stated that in regard to claims 1-5, 19-23, and 37-41, Somorjai et al. teach obtaining magnetic resonance spectra from biomedical samples (page 257, column 2), subdividing regions into subregions (page 258, column 1), developing classifiers for training sets, and using cross validation on the training sets, and allocating members of a test set to a certain class (page 258, column 2). The Examiner stated that this procedure is repeated a plurality of time (page 258, column 2, that training sets consisted of eight normal samples and eight unknown samples, and that training results on different subregions were assessed and aggregated for a consensus based upon majority scores (page 260, column 2).

The Examiner stated that Somorjai et al. do not teach the use of this method for identification and classification of bacterial species (present in claims 6-9, 24-27, 35, 36, 42-45, 53, and 54), however, Delpassand et al. et al. do show that NMR is a useful technique to rapidly identify pathogens, including bacteria, by providing a "fingerprint" within the proton spectrum (see entire

article). The Examiner stated that it would have been prima facie obvious to one of ordinary skill in the art at the time of the invention to use the method and classifier of Somorjai et al. to identify and classify bacteria (as was shown by Delpassand et al. to be distinguishable through NMR spectra). The Examiner stated that one would have been motivated to do so because Somorjai et al. explicitly stated that, although the example in the paper is directed to the identification of thyroid neoplasms, it could be used for any biomedical data. The Examiner stated that Somorjai et al. state that "we introduce and apply a new classification strategy called computerized consensus diagnosis. Its purpose is to provide a robust, reliable classification of biomedical data (see abstract)".

Applicants respectfully traverse the rejection made under on obviousness grounds based on the Delpassand et al and Somorjai references.

The Delpassand reference discusses obtaining MR spectra from only a few widely different genera of bacteria and identified the different genera by only visual inspection. Delpassand does not disclose the ability to identify different species of microorganisms within a genus. The presently claimed invention of claims 1, 19 and 37 provides for identifying the species of the microorganism within a genus as being different from other species within the same genus.

The present invention enables identification of microorganisms belonging to a broad range of both closely and distantly related microbial species.

Applicant urges that it would not have been obvious to combine Delpassand's approach with the SCS method of Somorjai because the successful introduction of SCS to the identification of bacteria requires the accumulation of a large data set of closely and distantly related organisms to establish that the present invention works, and such methodology would not have occurred to one of ordinary skill in the art. The Delpassand reference used only statistically non-significant number of strains, used only visual inspection, and did not disclose a way to identify species from other species within the same genus. Further the SCS method of Somorjai did not disclose the concept of discriminating closely and distantly related species.

In the Office Action, the Examiner stated that reference numbers 29 and 60 from the Information Disclosure Statement filed September 29, 2003 were not considered allegedly because copies were not provided. Applicant's file copy of the Information Disclosure Statement indicates that copies of these reference numbers were provided. In any case, applicant submits additional copies of these reference numbers 29 and 60 and relists them on a PTO-1449 form attached hereto under Exhibit B.

Applicant also wishes to inform the Examiner of a PCT search report issued in the corresponding PCT application which was filed in Canada. A copy of the PCT Search Report is enclosed. The prior art cited is already of record in this application. Applicant also submits a copy of the PCT Written Opinion and the Response to the Written Opinion. All of these documents are attached under Exhibit C.

Applicant hereby submits a check for $180 in accordance with 37 C.F.R. §1.98(c)(2) and §1,17(p).

No fee is believed to be due in connection with this Amendment. If any additional fee is required, authorization is hereby given to charge the amount of any such fee to Deposit Account Number 03-3125.

Respectfully submitted,

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I hereby certify that this correspondence is being deposited this date with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to:

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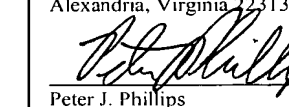

12/30/04

Peter J. Phillips Date
Reg. No. 29,691

Amendments to the Drawings

No amendments are proposed to the drawings.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a statistical classifier for enabling the ~~identificaitn~~ identification, preferably down to the species group or species level, of various microorganisms. As used herein, the term "microorganism" means any microscopic organism (i.e., any unicellular or multicellular living entity) including bacteria, fungi, parasites, viruses, protozoa and algae.

According to the present invention a one-dimensional ^{1}H MR spectrum of a microorganism such as a bacterial cell suspension provides an overview of hydrogen-containing compounds. Consequently, the ^{1}H MR spectrum is more representative of the physiology of the cell (metabolite pools) than its structure (comprising immobile components such as the cell wall). While many different bacterial groups may express and utilize essentially identical metabolic pathways, differing levels of enzyme expression and activity in different groups could give rise to distinctly different levels of particular metabolities when dissimilar groups are grown in similar environments. It was, therefore, proposed that significantly different metabolite pool sizes could be detected as differences between the ^{1}H MR spectra of the different bacterial groups. This was suggested in a previous study comparing selected bacterial ^{1}H MR spectra (5), however the small number of isolates examined and the qualitative identification methods described in that study did not permit automation or quantitative comparison of the species groups.

Form PTO-1449 U.S. Department of Commerce Patent and Trademark Office	Atty. Docket No. 62620/PJP	Serial No. 10/081,838
INFORMATION DISCLOSURE STATEMENT (Use several sheets if necessary)	Applicant Tania C. Sorrell et al.	
	Filing Date February 21, 2002	Group

U.S. PATENT DOCUMENTS

Examiner Initial		Document Number	Date	Name	Class	Subclass	Filing Date if Appropriate

FOREIGN PATENT DOCUMENTS

		Document Number	Date	Country	Class	Subclass	Translation	
							Yes	No

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

	1	Fujita NK, Reynard M, Sapico FL, Guze LB, Edwards JE Jr. Crytococcal intracerebral mass lesions: the role of computed tomography and nonsurgical management. Ann Intern Med (1981); 94:382-388
	2	Thevelein JM. Regulation of trehalose metabolism and its relevance to cell growth and function. 395-420. In Brambl R, Marzluf, GA, eds. The Mycota. Vol 3. Berlin: Springer Verlag Chapter 19, pp 395-420 (1996)

EXAMINER	DATE CONSIDERED

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this from with next communication to applicant.

CLINICAL REVIEW

29

Cryptococcal Intracerebral Mass Lesions

The Role of Computed Tomography and Nonsurgical Management

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Cephalic computed tomography (CT) is a sensitive technique for defining certain intracerebral diseases. Four patients with cryptococcosis were evaluated with cephalic computed tomography over 3 years. All had focal, intracerebral "contrast-enhanced" lesions consistent with cryptococcal mass lesions, confirmed histopathologically in two. An analysis of 55 cases of cryptococcal intracerebral mass lesions from the literature showed that 18% of patients with these lesions associated with cryptococcal meningitis did not have specific symptoms or signs of focal intracerebral disease or increased intracranial pressure. We therefore recommend that patients with cryptococcal meningitis, regardless of localizing symptoms or signs, be considered for cephalic CT evaluation to ascertain the presence of mass lesions. Three patients in this report were treated with systemic antifungal medication without surgery. Decreased size or disappearance of these lesions was seen on sequential CT scan in all patients. We conclude that selected patients with cryptococcal intracerebral mass lesions may be managed successfully with systemic antifungal therapy alone.

CRYPTOCOCCAL intracerebral mass lesion is a rare form of central nervous system infection due to *Cryptococcus neoformans.* Between June 1976 and July 1979 we treated four patients with cryptococcosis who had computed tomography (CT) abnormalities (focal, "contrast-enhanced" areas) compatible with intracerebral mass lesions. Histopathologic confirmation was obtained in two patients.

The most efficacious management of cryptococcal intracerebral mass lesions has not been established. Surgical resection has been done successfully in some patients (1-13). Of our four patients with cryptococcal intracerebral mass lesions, only one was treated with surgical resection; the other three were treated with systemic antifungal medications alone. We report these four cases and review the reports in the English literature of cryptococcal intracerebral mass lesions to further define this clinical entity and critically evaluate current methods of diagnosis and management.

Case Reports

CASE 1

A 27-year-old, black female heroin addict was admitted to the hospital in June 1978 for evaluation of numbness, weakness, and focal seizures of her left arm, severe bifrontal headache, and dry cough of 1 month's duration. She was alert and had normal vital signs. There were rales and wheezes over the right lower lung field. Weakness and spasticity of the left arm and accentuation of the deep tendon reflexes were present. Abnormalities consistent with a right lower lobe infiltrate were seen on the chest roentgenogram; skull roentgenograms were normal. Two contrast-enhanced areas (2.4 cm in the right frontoparietal and 1.3 cm in the left frontal lobes) consistent with mass lesions were present on the cephalic CT scan (Figure 1a). Both lesions were avascular on the cerebral angiogram.

A biopsy specimen of the right frontoparietal lesion had histopathologic findings compatible with a cryptococcal mass lesion. *Cryptococcus neoformans* was cultured from the biopsy specimen and cerebrospinal fluid from the lumbar region. The patient was begun on amphotericin B (0.3 mg/kg of body weight per day) intravenously and flucytosine (150 mg/kg · d) orally.

The patient's neurologic symptoms improved. However, the cephalic CT scan done 2½ months after admission showed three additional intracerebral lesions. Intravenous amphotericin B was increased to 0.6 mg/kg of body weight per day.

Three months after admission the patient became lethargic and spasticity of her left arm increased. An Ommaya reservoir was inserted for intraventricular administration of amphotericin B. Intravenous and intraventricular amphotericin B were given to cumulative doses of 3.75 g and 18 mg, respectively. A cumulative dose of 1147 g of flucytosine was also given. At the end of therapy (6 months after admission) a decrease in size of all mass lesions was noted (Figure 1b).

Twelve months after admission, the right frontoparietal lobe lesion was 1.1 cm and the left frontal lobe lesion 0.8 cm in diameter (Figure 1c). Eighteen months after diagnosis the patient's only residual defect was slightly decreased strength of her left hand.

Comment: Multiple cryptococcal intracerebral mass lesions associated with meningitis developed in this apparently normal host. The number of lesions increased from two to five during combined amphotericin B and flucytosine therapy. However, with intensification of medical therapy, decreased size of all lesions and improved strength were noted.

CASE 2

A 19-year-old Mexican woman was admitted to the hospital in May 1978 because of dizziness, unsteady gait, dysarthria, and impaired vision for 2 weeks. Four months before admission she was treated for pneumonia.

She was alert and had normal vital signs. Breath sounds were decreased in the right lower lung field. Gynecologic examination showed findings consistent with a 6-month intrauterine pregnancy. Findings of neurologic examination were normal.

An abnormality consistent with a tumor-like mass in the right lower lung field was present on the thoracic CT scan, and a 2-cm contrast-enhanced area in the right cerebellum was seen

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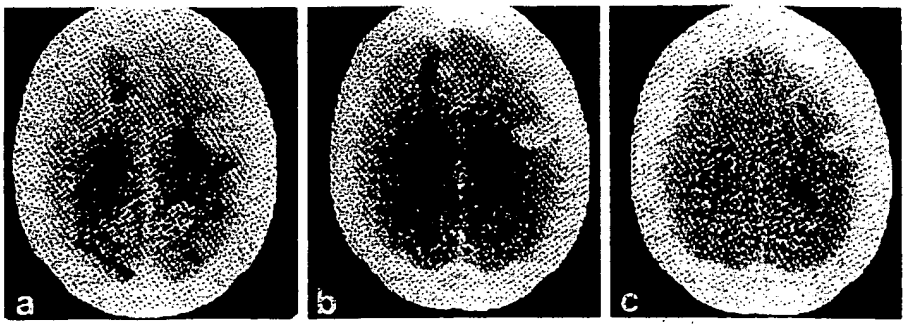

Figure 1. Serial computed tomography from Case 1. a. At time of diagnosis. b. At 6 months after diagnosis (termination of treatment). c. At 12½ months after diagnosis. Arrow points to the artifact from the catheter of Ommay reservoir.

on the cephalic CT scan. At thoracotomy, a gelatinous mass lesion containing *C. neoformans* involving the right middle and lower lobes was resected. Subsequent cerebrospinal fluid examination and culture showed cryptococcal meningitis. Cryptococcal antigen and antibody in serum were positive at dilutions of 1:1024 and 1:2, respectively. Amphotericin B therapy was initiated at 50 mg (approximately 0.7 mg/kg of body weight per day) intravenously.

One month after admission, after delivery of a premature infant, the patient became progressively lethargic and developed bilateral papilledema. Flucytosine (150 mg/kg • d orally) was added to the amphotericin B (0.3 mg/kg • d) regimen. Two months after admission, two new contrast-enhanced areas, both less than 0.5 cm in diameter, were seen on CT scan in the left caudate and left brain stem regions. Amphotericin B dosage was increased to 0.7 mg/kg • d, and an Ommaya reservoir was placed for intraventricular administration of amphotericin B.

A ventriculoperitoneal shunt was placed 5 months after admission because of development of hydrocephalus. At this time the cryptococcal antigen was positive in undiluted cerebrospinal fluid from the lumbar region. Six months after admission, the therapy was changed to miconazole (600 mg every 8 hours) intravenously and (20 mg every 24 hours) intraventricularly because of the lack of improvement and the presence of cryptococcal antigen in the lumbar cerebrospinal fluid at a dilution of 1:64. On the fifth day of this new regimen the Ommaya reservoir was removed because of evidence of a bacterial infection. The patient became fully alert after a 5-month course of intravenous miconazole therapy. The cryptococcal antigen in the cerebrospinal fluid and serum at the end of therapy was positive at dilutions of 1:4 and 1:8, respectively. She continued to do well 12 months after the termination of therapy (2 years after diagnosis). The cerebellar lesion diminished to 0.6 cm, and the caudate and brain stem lesions were no longer present.

Comment: Similar to the events in Case 1, new contrast-enhanced lesions consistent with cryptococcal intracerebral mass lesions appeared on CT scan during therapy and required intensification of medical therapy. The patient's continued poor condition and rising cryptococcal antigen titer in the cerebrospinal fluid prompted a change in therapy to miconazole, to which she responded.

CASE 3

(This case, reported previously by Sapico [14], was included in this report to show the sequential CT findings with treatment.) A 61-year-old black man with a history of chronic alcoholism was admitted to the hospital semicomatose with a temperature of 39.5 °C. The neck was supple; the liver was 14 cm in span. A right hemiparesis was present.

Cryptococcus neoformans was cultured from the cerebrospinal fluid from the lumbar area. The cryptococcal antigen in the cerebrospinal fluid was positive at a dilution of 1:128. Diffuse slowing was present on the electroencephalogram, and the technetium pertechnetate brain scan was normal.

On intravenous amphotericin B therapy the patient became afebrile and alert. The cerebrospinal fluid cryptococcal antigen was detectable at a dilution of 1:32 by the 19th day of therapy. Two months after admission a right homonymous hemianopia was detected. On the cephalic CT scan a 2.6- by 1.9-cm contrast-enhanced area with circumferential lucency was seen in the left parasagittal region of the occipital lobe (Figure 2a). Intravenous amphotericin B therapy was continued. Partial resolution of the lesion was documented on a second cephalic CT scan done 3 months after admission (Figure 2b). Complete resolution of the lesion was noted on the third cephalic CT scan done 4½ months after admission (Figure 2c). The hemianopia resolved completely.

Intravenous amphotericin B therapy was administered to a total dose of 3 g. The patient continued to do well, and the cerebrospinal cryptococcal antigen was positive at a dilution of 1:2 at 5 months after the beginning of therapy.

Comment: This alcoholic patient with cryptococcal meningitis developed a hemianopia two months after initiation of amphotericin B therapy. Contrast-enhanced area consistent with a cryptococcal mass lesion was present on cephalic computed tomography. Continued therapy with amphotericin B to a cumulative dose of 3 g resulted in complete resolution of the hemianopia and intracerebral abnormality.

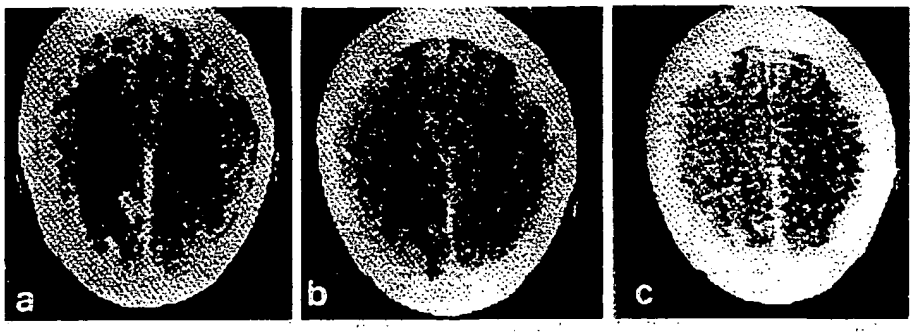

Figure 2. Serial computed tomography from Case 3. a. At 2 months after start of therapy for cryptococcal meningitis when hemianopia was detected. b. At 3 months of therapy. c. At termination of therapy (4½ months). Arrow points to lesion.

CASE 4

A 40-year-old black man was admitted to the hospital in October 1977 for evaluation of intermittent involuntary "twitching" of the left side of his face of 1 month's duration. Six weeks before admission he was treated for pneumonia.

He appeared well and had normal vital signs. There were rales at the left chest base; a slight unsteadiness was present on the finger to nose test in the left upper extremity. Evidence of a left lower lobe dense infiltrate was seen on the chest roentgenogram. Skull roentgenograms were normal. A 2-cm area of uptake in the right parietal lobe was seen on the radionuclide brain scan. Areas of abnormally increased uptake in the left lower lung field and the right cerebral hemisphere were present on the gallium scan. Two focal contrast-enhanced areas in the brain were present on CT scan. The larger right, frontoparietal lobe lesion was 2.0 cm in diameter. The electroencephalogram was normal.

Diagnostic thoracotomy showed a cryptococcal pulmonary mass lesion, which was resected. The lumbar puncture was normal; no cryptococcal antigen was detected in the cerebrospinal fluid. The serum cryptococcal antigen was positive at a dilution of 1:4 with no cryptococcal antibody present.

The patient was treated with a combination of amphotericin B (0.3 mg/kg of body weight per day) intravenously and flucytosine (150 mg/kg · d) orally. He received a total of 1.3 g of amphotericin B before moving to another state, where he received further evaluation and therapy. Four months after diagnosis, three focal contrast-enhanced areas were present on cephalic CT scan (Figure 3a); the right frontoparietal lesion was 2.2 cm in diameter. Craniotomy was done, and an approximately 2.5-cm hard, nodular yellow intracerebral mass was resected from the right frontoparietal area. Extensive reactive gliosis, numerous foamy macrophages, and organisms compatible with *C. neoformans* were present on histopathologic examination. Postoperatively a 2-week course of amphotericin B and flucytosine was given.

Focal seizures recurred 12½ months after the initial diagnosis, and the patient was given a third course of combined amphotericin B and flucytosine therapy followed by flucytosine alone for 3 months. The remaining two intracerebral lesions gradually diminished in size (Figure 2b), and no contrast-enhanced lesions were present on the last CT scan taken 22 months after the initial diagnosis (Figure 3c). The patient's only deficit was a mild left hemiparesis.

Comment: This patient had no conditions predisposing to the development of cryptococcosis. He did not have an associated meningitis. Two intracerebral contrast-enhanced areas consistent with cryptococcal mass lesions were detected on the initial CT scan, and a third was detected 4 months later. After surgical removal of the largest lesions the other two disappeared with further medical therapy.

Materials and Methods

All cases of central nervous system cryptococcosis of any type reported between January 1957 and December 1979 were reviewed with special attention to the presence of mass lesions. Moreover, all cases of cryptococcal intracerebral mass lesion before 1957, as referenced in previous publications, were evaluated (6-8, 15-30). Cases were included in this analysis if the following criteria were met: The mass lesions had a diameter of 1 cm or greater (an arbitrarily determined size) by measurement or estimation of surgical autopsy specimen; and the surgical or autopsy specimen had characteristic histopathologic changes of *C. neoformans* infection with organisms present (identified by fungal stains, culture, or both). The following variables were analyzed: patient characteristics, symptoms and signs, morphologic types of mass lesions, associated organ involvement, diagnostic techniques, and treatment.

Results

By the above listed criteria, 55 cases of cryptococcal intracerebral mass lesions were identified and included in the analyses (1-13, 15-48). Thirty cases were diagnosed during life; 25 cases were diagnosed at autopsy. Seven cases of probable cryptococcal intracerebral mass lesion were excluded from the analyses because of inadequate descriptions of autopsy or surgical specimens (49-55).

The age range was from 4 to 72 years with the median age of 42; only six patients were under 20 years old. Forty-three of 54 patients (80%) were men (sex was not mentioned in one patient). Race was stated in 29 patients; 19 were white, three black, and seven Oriental or Indian. Conditions predisposing to the development of cryptococcal meningitis, as outlined by Butler and associates (56), were found in only three (5%) of 55 cases; diabetes mellitus was found in two patients and sarcoidosis in one. One of the two patients with diabetes mellitus also had bullous pemphigoid and was under therapy with prednisone (20 mg/d) for 4 years. Other possible predisposing conditions existed in six additional patients and included pregnancy (four), alcoholism (one), and pulmonary tuberculosis (one).

A wide spectrum of nervous system symptoms was reported; many were attributed to the associated "chronic" meningitis. The commonest complaint was headache, which was present in 40 patients (73%). Less frequently encountered symptoms were mental status changes (confusion, emotional lability, lethargy, and coma); weakness; seizures; nausea, vomiting, or both; visual disturbances; and unsteady gait, each occurring in less than 33% of the patients. In two (4%) patients, no symptoms of central nervous system disease were noted, and intracerebral mass lesions compatible with cryptococcal mass lesions were found incidentally at autopsy (25). A third case

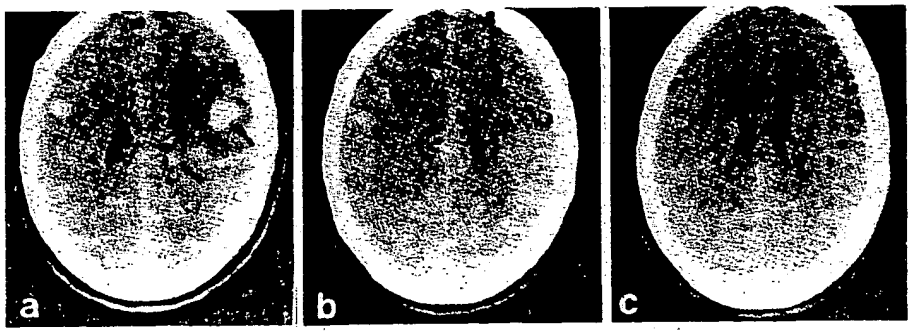

Figure 3. Serial computed tomography from Case 4. a. At 4½ months after diagnosis. b. At 15 months after diagnosis. c. At 22½ months after diagnosis. Arrows point to lesions.

Table 1. Cerebral Angiographic Abnormalities in Cryptococcal Intracerebral Mass Lesions (24 Patients)

Result		Patients
		n
Normal		1
Inconclusive		1
Abnormal		22
Avascular mass	15	
"Tumor shadow"	1	
Vessel shift	4	
Hydrocephalus	1	
"Swollen thalami"	1	
		24

from that report was omitted from our analysis because the mass lesion measured only 6 mm in diameter. These three patients died of unrelated illnesses.

The most frequent abnormalities detected on physical examination included focal weaknesses or hemiparesis (38%), papilledema (29%), cranial nerve abnormalities (27%), abnormal mental status (20%), and deep tendon reflex abnormalities (21%). Less frequently encountered physical abnormalities were nystagmus, ataxia, aphasia, and sensory deficits. Of note, six (11%) of 55 had normal findings on neurologic examination.

Among 34 patients who had an associated meningitis with cryptococcal intracerebral mass lesion, six had no characteristic symptoms or signs of a focal central nervous system lesion or increased intracranial pressure. No abnormalities of the neurologic examination were described in four patients, although one subsequently developed optic atrophy. The fifth patient had lethargy and bilaterally positive Babinski reflex, and the sixth patient had generalized hyperreflexia. Among 18 patients with mass lesions without meningitis, two discussed previously had no premortem evidence of any central nervous system disease.

Four basic morphologic types of cryptococcal intracerebral mass lesions were reported: abscess (9%), "gelatinous" mass (24%), "fibrogranulomatous" mass (15%), and mixed-type (43%). Four mass lesions (9%) could not be classified because of inadequate descriptions in the case reports, and one was a hemorrhagic granulomatous lesion. A cryptococcal intracerebral abscess resembled a well-developed pyogenic abscess and had a defined fibrous capsule with an inner fluid material containing variable numbers of cryptococcal organisms, inflammatory cells, and cellular debris. The gelatinous mass lesion was predominantly a mass of cryptococcal organisms with a thin reticular network of residual nerve and connective tissue with minimal to no inflammatory reaction. The fibrogranulomatous type was a firm mass with various degrees of fibrous and granulomatous reaction, mononuclear cell infiltration, and scattered cryptococcal organisms. The mixed type contained at least two of the three previously mentioned morphologic types.

Nineteen (35%) patients were found to have multiple lesions. The range of size was wide with each morphologic type. Of the 48 cases in which size was specified (either direct measurements or comparison to an object, for example, a hen's egg), 22 lesions were between 1 and 2.9 cm, 19 were between 3 and 5.9 cm, and seven were 6 cm in diameter or greater. In seven cases, the size of the intracerebral mass lesion was not specified; however, descriptions suggested a diameter greater than 1 cm.

The mass lesions were frequently associated with meningitis and pulmonary involvement. Cryptococcal meningitis was present in 34 (63%) of 52 cases (meningeal involvement could not be discerned in three cases), and pulmonary cryptococcis occurred in 15 of 29 autopsied cases and in two of 15 surviving patients. The existence of pulmonary involvement could not be ascertained in 13 cases. Other involved areas included the skin, bone, lymph nodes, and kidney. Each occurred in fewer than 10% of the total number of cases.

A number of diagnostic techniques were used to evaluate these patients. Skull roentgenograms were described in 22 cases, in which 12 were abnormal. Specific abnormalities of focal mass lesions were present in four patients; two with lateral pineal gland shift and two with focal intracerebral calcifications. Electroencephalogram was reported in eight cases; one was normal, six had focal abnormalities, and one had a diffuse abnormality. Among nine radionuclide brain scans, uptake in the area of the cryptococcal mass lesion was abnormal in all (all four morphologic types were represented). Abnormalities in cerebral angiograms were observed in 22 of 24 patients. The most frequently described abnormalities were "avascular mass" and "vessel shift" (Table 1). Ventriculography was done in 13 patients; four were normal, one was unsuccessful, and eight had findings compatible with either intraparenchymal or intraventricular mass lesion. Of the four pneumoencephalograms, a ventricular filling abnormality was found in three. Cephalic CT scan was done in two cases (3, 14); one of them was included in our case reports (Case 3). Focal, homogeneous contrast-enhanced areas were present in both.

All patients but one (43) with cryptococcal intracerebral mass lesions diagnosed during life, excluding our cases, underwent a resection of the lesion. Among the 29 cases, 13 patients were alive at the time of the published case reports (Table 2). The follow-up period ranged from 2 months to 6 years with a median of 1 year. Seven of the survivors had residual neurologic deficits, which included

Table 2. Comparison of Survivors and Nonsurvivors After Surgical Resection of Intracerebral Mass Lesions

	Survivors (n = 13)	Nonsurvivors (n = 16)
Age (median), *yrs*	33	49
Size of lesion (mean),* *cm*	4.2	3.8
Patients with multiple lesions, *n*	3	4
Patients with underlying disease, *n*	1	2
Patients with meningitis, *n*	4	9†
Patients given adjunctive antifungal medication, *n*	7	7

* If multiple lesions existed, only the largest one was tabulated.
† Existence of meningitis could not be ascertained in three of the 16 case reports; meningitis existed in nine of 13 nonsurvivors.

hemiparesis, homonymous hemianopia, and spasticity of extremities. Among the nonsurvivors, 10 patients died of causes not solely attributable to cryptococcosis (such as sepsis).

Poor prognostic factors of surgical outcome included advanced age and existence of an associated meningitis (Table 2). Factors that appeared not to affect outcome were size of mass lesions, existence of multiple mass lesions, and administration of effective antifungal medication. However, the six patients who survived the surgical procedure without adjunctive medication did not have meningitis. Although difficult to accurately assess, the clinical condition before surgery of the nonsurvivors as a whole seemed worse than that of the survivors. The distribution of the morphologic types among survivors and nonsurvivors was comparable.

Three patients with presumed cryptococcal intracerebral mass lesions were initially treated medically without surgery (1, 3, 43). All received amphotericin B intravenously, and one received a cumulative dose of 4.5 mg of amphotericin B intrathecally. One patient whose mass lesion was diagnosed on his second relapse died during the third course of anticryptococcal therapy (43). Two patients had neurosurgical resection of the mass because of the lack of resolution after a full course of amphotericin B therapy (2 months of intravenous therapy in one patient and 3 g total in the other). Both patients were cured after surgery. In both cases, *C. neoformans* grew from large resected mass lesions; one measured 6 cm in diameter and the other, 4 by 3 by 3 cm.

In the other 25 cases, cryptococcal intracerebral mass lesions were not suspected pre-mortem and discovered only at the time of autopsy. Eighteen patients were not treated with amphotericin B or flucytosine because cryptococcosis was not suspected before death or the medications were not available. The other seven patients were treated for cryptococcal meningitis (31, 33, 36, 38, 41, 44). Six died during the initial course of therapy, and the seventh patient had an initial response but subsequently had a relapse and died. Within this latter group, all had mass lesions under 3 cm in diameter, and six had multiple lesions.

Discussion

The exact incidence of cryptococcal intracerebral mass lesions is unknown. The most recent review indicates the existence of approximately 40 reports of cases of cryptococcal mass lesions of the brain and spinal cord in the literature (12). A comprehensive search in the English language literature to 1957 has uncovered cases of cryptococcal intracerebral mass lesions not tabulated in previous publications (4, 5, 31, 33-38, 40-44, 46-48). Other cases of intracerebral mass lesions were probably missed because autopsies were not done in many patients who died of cryptococcosis. Asymptomatic intracerebral mass lesions in patients who survived an episode of cryptococcal meningitis or disseminated cryptococcosis may also have been missed if appropriate diagnostic tests were not done.

The characteristics of patients with cryptococcal intracerebral mass lesions were similar to those of patients with cryptococcal meningitis except for the remarkably lower frequency of serious, underlying conditions (5% as compared to 50% in patients with cryptococcal meningitis [56]).

The symptoms and signs noted in these patients were due to the intracerebral mass lesions, meningitis, or both. Of importance is that 18% of patients with cryptococcal meningitis and intracerebral mass lesions had no specific symptoms or signs of focal lesions or increased intracranial pressure that necessitate evaluation for an intracranial mass lesion. For this reason and because data on the frequency of asymptomatic intracerebral mass lesions in patients with cryptococcal meningitis are limited, we suggest that all patients with cryptococcal meningitis, regardless of symptoms, be considered for diagnostic evaluation for these mass lesions. This suggestion is particularly relevant because of the current trend towards shorter courses of therapy for cryptococcal meningitis (57, 58), because patients with both meningitis and intracerebral mass lesions may need a longer course of therapy. Further diagnostic evaluation for cryptococcal intracerebral mass lesions in patients with nonmeningeal cryptococcosis (such as isolated pulmonary cryptococcosis without neurologic symptoms or signs) is less clear, as the number of cases available for analysis was small.

The most sensitive and accurate method of defining cryptococcal intracerebral mass lesions has not been determined. All available techniques had limitations related to insensitivity, nonspecificity, or invasiveness. In our analysis, skull roentgenograms were the least helpful. The electroencephalogram and the radionuclide brain scan, although not widely used, were more sensitive. However, brain scans missed smaller lesions as observed in our fourth case and as documented in the literature (47). A false-positive brain scan also has been reported (46). The major disadvantage of the brain scan was the inability to sharply outline the mass, so that an accurate assessment of size could not be done. The gallium scan was not used extensively but may have the same disadvantage as the brain scan. The most informative modality was the cerebral angiogram. However, the characteristic finding of an avascular mass was noted in only 68% of abnormal angiograms.

Only one other case of surgically doumented cryptococcal intracerebral mass lesion (excluding our cases) in which CT scan was done has been reported (3). A focal, homogeneous contrast-enhanced area similar in appearance to those seen in our four cases was described. In one of our patients (Case 1), the largest lesions initially had a "donut" configuration. A rim of decreased density probably representing circumferential edema surrounded many of these lesions. On the basis of these cases, cryptococcal intracerebral mass lesions appeared as focal homogeneous or donut-shaped, contrast-enhanced areas with or without accompanying circumferential area of decreased density on CT scan. Because of its apparent high sensitivity, satisfactory resolution, and noninvasive nature, CT scan appears to be the best modality for localization and enumeration of cryptococcal intracerebral mass lesions.

There were certain limitations to CT scan. The first limitation has been the nonspecificity of the focal, homogeneous contrast-enhanced lesion for cryptococcal mass lesion. A number of infectious entities have been reported that cause similar-appearing lesions and include pyogenic abscess (59, 60), nocardial abscess (60), tuberculous mass lesion (61), and aspergillus abscess (60). In addition, intracerebral hemorrhage and neoplasm have also been reported to cause contrast-enhanced areas (59).

The variability of the levels of the roentgenogram sections through the brain that occur from one study to another has been the second limitation of computed tomography. The sections are usually spaced 1.3 cm apart, possibly accounting for the absence of small lesions on the initial CT scan on three of our patients (Cases 1, 2, and 4) and their appearance on subsequent studies. This variability may cause an artifactual change in the size of mass lesions or artifactual disappearance of smaller lesions. Despite these limitations, computed tomography was extremely useful in our four patients in the detection of cryptococcal mass lesions, and in monitoring the responses to therapy.

The most efficacious management of the cryptococcal intracerebral mass lesion is difficult to decide from the review of the literature. We have tentatively concluded that "large" mass lesions (actual size greater than 3 cm in diameter) in surgically accessible areas most likely require resection for cure. However, for "smaller" lesions, medical therapy alone may be curative. In all four of our patients all lesions diminished in size or disappeared with medical therapy, either with amphotericin B alone or in combination with flucytosine. Why new lesions appeared during therapy in our four patients is unknown. Possible explanations include variations in the roentgenographic "cuts" from one study to the next, increase in inflammatory response to a previously undetected small lesion, and continued growth of an initially undetected lesion until eventual suppression by the antifungal medications.

The optimum length of medical therapy and the efficacy of systemic antifungal therapy in managing cryptococcal intracerebral mass lesions are unknown and require further study. The role of intrathecal administration of antifungal drugs for this disease is also unknown. Although medical therapy was favorable in our four patients, each patient must be considered individually with regard to medical versus surgical therapy, regardless of the size of the mass lesion. If medical therapy alone is initiated, close monitoring with serial cephalic computed tomography and serum cryptococcal antigen and antibody titers and a longer course of medical therapy are recommended. Recommendations for the use of high-dose corticosteroid therapy with systemic antifungal therapy in central nervous system cryptococcosis cannot be made at present because of limited data (1, 62-66).

Summary

Cryptococcal intracerebral mass lesion is an entity of unknown incidence. Most patients have specific symptoms or signs suggestive of a focal mass lesion or increased intracranial pressure, but a significant minority (approximately 20% of patients with both cryptococcal intracerebral mass lesion(s) and meningitis and 10% of patients with mass lesion(s) without meningitis) do not. Therefore, until experience dictates otherwise, we suggest that patients with cryptococcal meningitis be evaluated for the existence of intracerebral mass lesions regardless of symptoms or signs. Despite limitations, the CT scan appears to be the most suitable diagnostic and monitoring modality for cryptococcal intracerebral mass lesions. Preliminary evidence suggests that medical therapy alone may be adequate as the initial mode of therapy in patients with small mass lesions; however, each patient must receive individualized considerations on the type of therapy. If medical therapy alone is chosen, a prolonged course of systemic antifungal agents with close monitoring is recommended.

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The Mycota

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19 Regulation of Trehalose Metabolism and Its Relevance to cell Growth and Function

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CONTENTS

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I. Introduction

Trehalose is a disaccharide (α-D-glucopyranosyl α-D-glucopyranoside) commonly found in fungi and present at particularly high concentrations in resting cells and survival forms such as spores and sclerotia. Two specific lines of research with respect to trehalose have received much attention. The first is in control of trehalose mobilization during the initiation of growth in resting cells and, more recently, the possible role of trehalose as a stress protectant. With respect to trehalose mobilization in fungi, two mechanisms have been proposed to trigger its onset, depending on the type of trehalase present in a particular species.

For species containing an acid trehalase, decompartmentation between trehalose and trehalase was proposed as the triggering mechanism; for species with a trehalase regulated by cAMP-dependent protein phosphorylation, trehalose mobilization was suggested to be triggered by a cAMP-dependent protein phosphorylation cascade. Closer investigation of the latter process in the yeast *Saccharomyces cerevisiae* has resulted in considerable progress in the understanding of the mechanisms of nutrient-induced signal transduction. A remarkable outcome from these studies was the recent finding that a subunit of the trehalose-6-phosphate synthase/phosphatase complex is in some way involved in the control of glucose influx in glycolysis. This has generated intensive interest in this enzyme, which had earlier received only little attention. Since both the trehalase gene and the genes encoding the trehalose-6-phosphate synthase/phosphatase have now been cloned in yeast, rapid progress can be expected concerning the control of trehalose metabolism in yeast and also the identification of homologous genes in other fungi. The decompartmentation mechanism as initiator of trehalose mobilization has received very little support. On the contrary, several fungi which were supposed to

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have only an acid trehalase and for which this mechanism had been proposed as trigger for trehalose mobilization, appear also to have the neutral phosphorylated trehalase, or at least another trehalase, which most likely is the true initiator of rapid trehalose breakdown.

A second subject which has received considerable attention in recent years is the possible function of trehalose as stress protectant. Although the correlation between high trehalose content and strong gassing power had been known for a long time by baker's yeast producers, in particular for dried baker's yeast, the subject had received only little attention in fundamental research. Recently, a strong correlation between trehalose content and stress resistance has been demonstrated in yeast for a wide range of physiological conditions and by making use of an extensive series of mutants. Specific manipulation of the trehalose level, however, has only become possible with the recent cloning of the genes involved in trehalose metabolism. These studies are now in full progress. From the data already available, it appears that, at least in yeast, other mechanisms exist that are required for stress resistance and without which trehalose is not effective as stress protectant.

II. Occurrence, Accumulation, and Mobilization of Trehalose

The occurrence of trehalose is very widespread in fungi, in both vegetative and reproductive stages (Elbein 1974). Especially in reproductive stages, such as spores, and survival forms, such as sclerotia, the level of trehalose can be very high, much higher than that of other carbohydrates like glycogen or sugar alcohols. In many instances (e.g., in the ascospores of *Saccharomyces cerevisiae*) trehalose is virtually the only sugar present in the cytoplasm (Thevelein 1984c).

Intensive trehalose synthesis in fungi is observed not only during sporulation and differentiation processes but, more generally, during periods of reduced growth rate (e.g., during starvation in vegetative cells: Thevelein 1984c). *Saccharomyces cerevisiae* cells can continue to accumulate trehalose for many hours when fed with low sugar concentrations (Grba et al. 1975). This property is being used by commercial baker's yeast producers to enhance the trehalose content of the cells to up to 20% of the dry weight (Trivedi and Jacobson 1986; Gelinas et al. 1989; P. Van Dijck and J. Thevelein, unpubl. results). In the yeast *Saccharomyces cerevisiae*, reduced growth rate correlates well with higher trehalose content (Küenzi and Fiechter 1972) and cells starved for nitrogen, phosphate, or sulfate in the presence of glucose synthesize large amounts of trehalose (Lillie and Pringle 1980). In addition, incubation of yeast cells at sublethal temperatures, which also reduces the growth rate, induces strong trehalose accumulation (Grba et al. 1975, 1979; Hottiger et al. 1987b).

In general, resumption and stimulation of growth are associated with trehalose mobilization. This is particularly prominent during the induction of growth in spores and sclerotia, where the high trehalose content is usually rapidly mobilized during the initial stages of germination. A similar mobilization of trehalose is observed upon addition of nutrients to starved yeast cells (Thevelein 1984c). Prolonged starvation of trehalose-containing yeast cells also causes gradual, albeit very slow, mobilization of trehalose (Panek 1963; Lillie and Pringle 1980), a process which is also known to occur during storage of baker's yeast (Stewart et al. 1950; Suomalainen and Pfäffli 1961). Rapid trehalose mobilization has also been observed in glucose-limited self-synchronized chemostat cultures of *S. cerevisiae* during initiation of the budding phase of the cell cycle (Küenzi and Fiechter 1969; von Meyenburg 1969; Boiteux 1992). The correct interpretation of this phenomenon appears to be that the cells temporarily stay in the stationary G0 phase because of the glucose limitation and then, suddenly, triggered by an unknown factor, enter the G1 phase of the cell cycle in a synchronous way. Hence, mobilization of trehalose in this system is also associated with resumption of growth in stationary-phase cells, rather than with a specific phase of the cell cycle, as was concluded by Küenzi and Fiechter (1969).

III. Enzymes of Trehalose Metabolism

A. Two Types of Trehalases in Fungi

In a previous review, we divided the fungal trehalases into two types: a first type with an acid pH optimum and a high heat stability and a second type which displays a neutral pH optimum, a low heat stability, and is regulated by cAMP-depen-

dent protein phosphorylation (Thevelein 1984c). Because the acid trehalase appeared to be a purely hydrolytic lysosomal type of enzyme, while the neutral trehalase had a definite control itself over trehalose mobilization, we proposed to call the two types nonregulatory and regulatory trehalases, respectively. Although the division in the two types turned out to be most useful, the names acid trehalase and neutral trehalase have gained more widespread acceptance and will be used here.

The acid trehalase showed a widespread occurrence in fungi (Thevelein 1984c). Its presence in *Trichoderma* has been confirmed (Alabran et al. 1983) and it has also been reported in *Chaetomium* (Sumida et al. 1989) and *Humicola* (Zimmermann et al. 1990). With the exception of *Cunninghamella japonica*, a member of the Mucorales, claimed to be closely related to higher fungi (Tereshina et al. 1988), the acid trehalase has not been reported in zygomycetes. The neutral trehalase had only been found in the zygomycetes (*Phycomyces*, *Mucor*, and *Piptocephalis*) and in *Saccharomyces* (Thevelein 1984c) (but not in *Pichia pastoris*, which was erroneously named *Saccharomyces cerevisiae*). Since then, it has also been reported in *Candida* (Argüelles and Gacto 1985, 1986; Argüelles et al. 1986) and in *Schizosaccharomyces* (De Virgilio et al. 1990, 1991b; Carrillo et al. 1992).

In *S. cerevisiae*, both types of trehalase had been reported. Since then it has been well established that *S. cerevisiae* contains an acid trehalase in the vacuole that is processed from an inactive precursor by protease A in the same way as the other vacuolar hydrolases, and a neutral trehalase in the cytosol (Keller et al. 1982; Londesborough and Varimo 1984; Harris and Cotter 1987; Mittenbühler and Holzer 1988). The two trehalase types and the same subcellular distribution have also been reported for *Candida utilis* (Argüelles and Gacto 1988). In *Schizosaccharomyces*, the acid trehalase is located only in ascus cell walls (Inoue and Shimoda 1981a, b; De Virgilio et al. 1991b). The active phosphorylated form of neutral trehalase in *S. cerevisiae* appears to be a dimer composed of two identical subunits with MW 80000. Inactive trehalase has also been detected as forms with a MW of 80000 and 320000. The enzyme can be activated in vitro by phosphorylation and deactivated by dephosphorylation. Activation was shown to be associated with incorporation of about 1 mol of phosphate per mol of subunit. As opposed to acid trehalase, neutral trehalase is not a glycoprotein and requires Ca^{2+} or Mn^{2+} for activity (Ortiz et al. 1983; Uno et al. 1983; Londesborough and Varimo 1984; Dellamora-Ortiz et al. 1986; App and Holzer 1989). The properties of neutral trehalase in *Candida* are very similar, except that the enzyme appears to be a tetramer with a MW of 280000 (Argüelles and Gacto 1986; Argüelles et al. 1986). The gene encoding the *S. cerevisiae* neutral trehalase has recently been cloned (Kopp et al. 1993). The predicted amino acid sequence of the neutral trehalase contains one consensus site for cAMP-dependent protein phosphorylation. The gene shows homology to the periplasmic trehalase from *Escherichia coli* (Gutierrez et al. 1989) and to the rabbit small intestinal trehalase (Ruf et al. 1990). The availability of the neutral trehalase gene from yeast makes it possible to investigate the relationship between the different types of trehalases at the DNA level. It will also allow to investigate whether the neutral trehalase was not detected in other fungi because of its lability in cell extracts. Recent experiments in *Neurospora*, for instance, have made it very unlikely that acid trehalase would be responsible for the rapid changes in the trehalose level observed under several conditions (H. Terenzi, pers. comm.).

B. Trehalose-6-Phosphate Synthase and Phosphatase

The enzymes responsible for trehalose synthesis have been studied in *Dictyostelium* in relation to the onset of trehalose synthesis during sporulation and more extensively in the yeasts *S. cerevisiae* and *Candida utilis*. In *Dictyostelium* the presence of an inhibitor masks trehalose-6-phosphate synthase activity in extracts of cells from early developmental stages. The identity of this inhibitor has not been studied further. The activity of the synthase reaches a maximum at the preculmination stage, i.e., about 17–18 h after the initiation of starvation of the myxamoebae, and remains constant in subsequent developmental stages (Roth and Sussman 1968; Killick and Wright 1972a,b).

In *S. cerevisiae*, the activities of trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase copurify (Cabib and Leloir 1958; Vandercammen et al. 1989; Londesborough and

Vuorio 1991; Bell et al. 1992). Both activities reside in one complex, suggesting that the trehalose-6-phosphate might be channeled inside the complex rather than released into the cytosol. The purified complex, which displays both activities, has a total MW of 600–800 kDa and appears to consist of three subunits with MW of 130, 100, and 56 kDa, respectively. In the absence of strong protease inhibitors, subunits are obtained with lower MW. Partial proteolysis also abolishes the strong activation of trehalose-6-phosphate synthase by fructose-6-phosphate (Vuorio et al. 1992). Phosphate acts as an inhibitor of trehalose-6-phosphate synthase activity but stimulates trehalose-6-phosphate phosphatase activity (Vandercammen et al. 1989; Londesborough and Vuorio 1991; Vuorio et al. 1992). Partial proteolysis has been reported to activate both the *S. cerevisiae* (Londesborough and Vuorio 1991) and the *Candida utilis* (Vicente-Soler et al. 1989) enzyme. The genes encoding the three subunits have been cloned and sequenced. Most remarkably, the *TSS1* (= *TPS1*) gene, which encodes the smallest subunit, turned out to be identical to the *GGS1* (= *FDP1* = *BYP1* = *CIF1*) gene (Bell et al. 1992; Vuorio et al. 1992). The *GGS1* gene was cloned independently by complementation of the *fdp1*, *byp1*, and *cif1* mutants which show a specific growth defect on rapidly fermented sugars, like glucose, fructose, mannose, and sucrose (Gonzalez et al. 1992; Hohmann et al. 1992; Thevelein 1992; Van Aelst et al. 1993). The *GGS1/TSS1* gene displays significant homology with part of the *TSL1* gene which encodes the 130-kDa subunit (37% identity at the amino acid level). Truncation of the *TSL1* gene, resulting in a MW decrease of the protein from 130 to 90 kDa, causes insensitivity to the allosteric regulators fructose-6-phosphate and phosphate, an effect similar to that of partial proteolysis (Vuorio et al. 1992, *TSL1* sequence: EMBL X72788). The recent cloning and deletion of the *TPS2* gene encoding the 100-kDa subunit indicate that it probably encodes the trehalose-6-phosphate phosphatase (De Virgilio et al. 1993). A temperature-sensitive mutant apparently defective in trehalose-6-phosphate phosphatase was isolated several years ago (Piper and Lockheart 1988) but it is unclear whether it is affected in the 100-kDa subunit. Recently, an additional gene, now called *TPS3*, has been entered in the EMBL gene bank (EMBL M88172). The predicted amino acid sequence shows 63% identity to the amino acid sequence of the *TSL1* gene product over a large stretch of 798 aa. Whether *TPS3* represents a second gene encoding the large 130-kDa subunit is not yet clear. Remarkably, both the *TPS2* and *TPS3* genes also show homology to *GGS1/TPS1*. The exact function of the four proteins encoded respectively by *GGS1/TPS1*, *TSL1*, *TPS2*, and *TPS3* will have to become clear in the future. Initial results available at present appear to indicate that *GGS1/TPS1* is responsible for the main trehalose-6-phosphate synthase activity. Deletion of *GGS1/TPS1* abolishes trehalose-6-phosphate synthase activity (Bell et al. 1992) and expression of *GGS1/TPS1* in *Escherichia coli otsA* mutants which are defective in trehalose-6-phosphate synthase activity restores trehalose accumulation (McDougall et al. 1993).

It was earlier suggested that *S. cerevisiae* contains two types of trehalose-6-phosphate synthase, the first type using UDPG, whereas the second type would use ADPG and would be maltose-inducible (Paschoalin et al. 1989). This claim originated from the observation that the *fdp1* and allelic *ssi1* mutants were unable to synthesize trehalose under normal conditions. Only when incubated with maltose or when a constitutive *MAL* gene (*MAL*^{c}) was present in the strain, was trehalose accumulation observed (Panek et al. 1980; Operti et al. 1982). The recent cloning of the genes encoding the UDPG-linked trehalose-6-phosphate synthase will facilitate a definite solution to this problem. On Southern blots, however, no close homologues of *GGS1/TPS1* are detected (Van Aelst et al. 1993). Whether the other genes, *TSL1*, *TPS2*, and *TPS3*, play a role in maltose-induced trehalose accumulation remains to be investigated.

In *Candida utilis*, two UDPG-utilizing trehalose-6-phosphate synthase enzymes have been reported (Vicente-Soler et al. 1989). One of the two enzymes was suggested to be regulated by phosphorylation (see below).

IV. Transport of Trehalose

Few data are available on transport of trehalose in fungi. In *S. cerevisiae* trehalose transport has been described by Kotyk and Michaljanicova (1979), who presented evidence for a trehalose/H$^+$ symport mechanism. More recently, Crowe et al. (1991) showed that in *S. cerevisiae* trehalose transport becomes derepressed upon glucose exhaus-

tion, while addition of glucose to stationary-phase cells results in a loss of trehalose transport, which requires protein synthesis for reversibility. They also investigated trehalose transport in isolated membrane vesicles and obtained in this way new evidence for trehalose/H^+ symport (De Araujo et al. 1991).

V. Functions of Trehalose

A. Trehalose as Storage Carbohydrate Versus Trehalose as Stress Protectant

The accumulation of trehalose during sporulation, its presence in large amounts in spores and resting cells, and its mobilization during the resumption of growth has always been taken as evidence for a role of trehalose as storage carbohydrate (Elbein 1974; Thevelein 1984c). In our review in 1984 we already pointed out that the high level of trehalose in spores was also believed to enhance the resistance of the spores against extreme environmental conditions, such as high temperature, freezing, desiccation, etc. Although the idea was put forward many years before (Sussman and Halvorson 1966) only one paper had been published supporting it. Emyanitoff and Wright (1979) showed that *Dictyostelium* spores with a higher trehalose level were more resistant to heat stress than spores with a lower trehalose level. It can be presumed, however, that at that time baker's yeast companies were already well aware of the correlation between trehalose and stress resistance of baker's yeast, in particular in connection with the production of "Instant Active Dry Yeast". This is commercial baker's yeast that not only remains active in dried form but also does not need rehydration prior to mixing with flour (Trivedi and Jacobson 1986). In 1961, Suomalainen and Pfäffli still reported a relatively low trehalose content of 8.5% of the dry weight for commercial baker's yeast, while at present values between 15 and 20% are common (Trivedi and Jacobson 1986; Gélinas et al. 1989; P. Van Dijck and J. Thevelein, unpubl results). An early publication by Pollock and Holmstrom (1951) already suggested a possible relationship between trehalose content and resistance against drying in baker's yeast.

The function of trehalose as stress protection metabolite has been strongly advocated in a review by Van Laere (1989). In a more recent review, Wiemken (1990) went even a step further and pleaded for dismissal of the idea that trehalose primarily functions as reserve carbohydrate. However, in their argumentation, both authors failed to consider a number of important data. Both authors argued, mainly based on the situation in yeast, that the synthesis and mobilization of trehalose does not fit with the behavior of a reserve substance. Trehalose is synthesized under conditions of starvation, also starvation for glucose, and mobilized when nutrients, especially glucose, are abundant. However, in yeast cells, glucose depletion is invariably followed by a huge increase in the glycogen content and resuspension of stationary-phase cells in glucose-containing medium also results in nearly complete disappearance of glycogen (Lillie and Pringle 1980; François et al. 1987, 1991). The changes in the glycogen content appear to be due, at least to an important extent, to the fact that the main glycogen synthase is repressed in the presence of a fermentable carbon source (Farkas et al. 1991; see also below). Exponentially growing yeast cells contain only little carbon reserves, and the better the substrate the less reserves they store. This is probably best explained by the fact that the survival of a microbial species is best supported by a maximal rate of proliferation under nutrient-rich conditions rather than expenditure of energy for storage of large amounts of carbohydrate reserves. In general however, yeast cells and other fungi do not only accumulate trehalose in resting cells. Growing cells of many fungal species (Elbein 1974) and also Streptomycetes (Braña et al. 1986) contain in addition to glycogen also trehalose in quantities which are most probably too low to act as effective stress protectant.

Another argument put forward by Van Laere (1989) and Wiemken (1990) was that during germination of *S. cerevisiae* ascospores and spores of other fungi, and also during growth induction in stationary-phase yeast cells resuspended in a glucose-containing nutrient medium, the supply of glucose from trehalose breakdown appears insignificant compared to glucose uptake from the medium (Barton et al. 1982; Van Laere 1986a). However, these studies were performed under laboratory conditions with spores germinating rapidly in liquid media under optimal conditions, i.e., with a large supply of external sugar. Under natural conditions, i.e., slow germination with limited external nutrients and on solid media, trehalose mobilization might easily prove to be

more important for the supply of energy and carbon to the germinating spore. The same reservation has to be made for the observation that during germination of certain fungal spores degradation products of trehalose leak into the medium, some of which, such as glycerol, appear to be energetically useless (Van Laere et al. 1987; Van Laere and Slegers 1987; Van Laere 1989). The "uselessness" of these secreted products is also less evident when the spores germinate on solid media compared to the liquid media used in standard laboratory germination experiments. In addition, these products may have less obvious functions, such as phosphate recovery in the case of glycerol as fermentation product. A system which might provide an interesting analogy is the self-synchronization of yeast cells in glucose-limited chemostats. Under glucose limitation yeast cells in a chemostat will spontaneously synchronize their cell cycles in such a way that the cells remain in the stationary phase G0 for a certain amount of time and then, suddenly, triggered by an unknown factor, exit the stationary phase and start synchronously with the next cell cycle. Exit of stationary phase is characterized by the same phenomena observed during induction of spore germination and growth induction in resting cells, i.e., rapid mobilization of trehalose, a burst in glycolytic and respiratory activity as evidenced by a sudden increase in CO_2 output, O_2 uptake, and medium acidification (Küenzi and Fiechter 1969; Von Meyenburg 1969; Boiteux 1992). The important difference, however, is that the glucose level in the medium in the glucose-limited chemostat culture is only in the micromolar range. During stationary phase exit it increases transiently because a small part of the glucose derived from trehalose breakdown leaks into the medium. Apparently, trehalose breakdown is so fast that glucose catabolism cannot follow. It is clear that in this case the burst in metabolism during the onset of budding is entirely due to glucose derived from trehalose breakdown rather than glucose taken up from the medium. Hence, the leakage of glucose derived from trehalose into the medium cannot be explained on the basis that trehalose mobilization has no function in carbon and energy provision. A similar situation might prevail during germination and growth induction under natural, glucose-limited conditions.

Another important aspect not considered by either Van Laere (1989) or Wiemken (1990) is that many fungal spores do not need exogenous nutrients for germination and are able to germinate in distilled water (Gottlieb 1978). Typical examples are *Neurospora* ascospores (Sussman 1954), *Dictyostelium* spores (Cotter 1975), and spores of *Cunninghamella* (Tereshina et al. 1988) which consume trehalose, the only storage carbohydrate they contain, during germination in distilled water. For *Cunninghamella* spores, it was reported that they use their trehalose stock at a reduced rate, when provided with exogenous glucose, and that mutants with a lower trehalose content and reduced trehalase activity showed a slower rate of spore germination (Tereshina et al. 1988). Other examples of spores that probably use their trehalose reserve as energy and carbon source during germination are sporangiospores of *Mucor* and *Pilobolus*. Their germination can be induced by nonmetabolizable glucose analogues in media lacking a carbon source (Tripp and Paznokas 1982; Bourret 1986). Panek and Bernardes (1983) also suggested that trehalose was important for supplying energy during germination of yeast ascospores, based on the reduced germination capacity of ascospores from the *sst1* mutant. This conclusion, however, seems somewhat preliminary, since *sst1* is allelic with *GGS1*/*TPS1* and all *ggs1*/*tps1* mutants, including *sst1*, show pleiotropic defects (e.g., a strongly reduced sporulation capacity; Panek and Bernardes 1983; Van Aelst et al. 1993).

It remains intriguing why stationary-phase yeast cells and yeast ascospores mobilize their large trehalose reserve when supplied with plenty exogenous glucose. This behavior is probably best understood by comparison with a completely different biological system displaying a similar behavior (i.e., mobilization of reserves upon supply of exogenous nutrients). Newly hatched chickens contain a yolk sac, the contents of which are rapidly used during initial development. However, when the newly hatched chickens are starved for food, they do not use the contents of the yolk sac and it remains present as such (V. Darras, pers. commun.). Why do the chickens use this reserve when they have food and not when they lack food? The answer in this case appears to be straightforward: when the chickens are fed, they start to develop and the resorption of the yolk sac is part of their developmental program. When they are starved, initiation of the developmental program is postponed and the reserve is not used for the time being. Hence, the presence of food does not trigger directly the use of the reserve, but

rather the start of a developmental program of which depletion of the reserve is a normal part. A similar conclusion might well be true in the case of germinating fungal spores and during resumption of growth in starved yeast cells. The presence of nutrients does not directly trigger the mobilization of the trehalose but triggers a developmental program, (i.e., germination or growth induction) of which the complete usage of the trehalose is only a part. When, for instance, this program is arrested, trehalose mobilization is also arrested. When yeast ascospores or stationary-phase yeast cells are given only glucose and no other nutrients, germination or growth induction is quickly arrested and also the mobilization of the trehalose is only transient. After some time the cells start to resynthesize trehalose from the glucose present in the medium. In this case, the argument put forward by Van Laere (1989) and Wiemken (1990) that the cells only synthesize trehalose when starved for external carbohydrate, clearly does not apply. Yeast cells starved for nitrogen or another essential nutrient in the presence of glucose accumulate large amounts of trehalose (Lillie and Pringle 1980). While spore germination is obviously a developmental program, induction of growth in stationary-phase cells and even the transition from slow-growing derepressed yeast cells to rapidly growing glucose-repressed yeast cells can be regarded as such. The nature of the mechanisms involved in triggering spore germination in *S. cerevisiae*, *Phycomyces blakesleeanus*, and *Mucor rouxii*, growth induction in stationary-phase cells of *S. cerevisiae*, and the transition from the slow-growing derepressed cell type in yeast to the rapidly growing glucose-repressed type supports this idea. The addition of glucose triggers in all these cell types a cAMP-dependent protein phosphorylation cascade which is believed to be responsible for, or at least stimulate, the induction of germination, the induction of growth, the switch in cell type, and the concurrent mobilization of trehalose (for review see Thevelein 1988). This supports the idea that glucose does not specifically trigger depletion of trehalose, but rather that the latter is only part of a developmental program initiated in the presence of glucose. A possible argument against this conclusion is that germination of yeast ascospores in acetate-containing medium occurs without mobilization of trehalose (Donnini et al. 1988). It should be emphasized, however, that under such conditions the cells remain glucose-derepressed (e.g., respiration fully active), whereas addition of glucose leads to glucose repression and a rapid switch to fermentative metabolism. Germination under conditions of glucose repression or derepression might not necessarily involve identical mechanisms. Obviously, it can also be argued that the yolk sac in young chickens is only a remnant of the yolk in the egg which is used by the developing chicken in the egg in the absence of any external food. The main function of the yolk reserve therefore would still be to supply food in the absence of external nutrients. The complete resorption in the developing young chicken would only constitute final consumption of anything left over. The same reasoning can be applied to the trehalose reserve in fungal and actinomycete spores and starved yeast cells. When incubated in the absence of nutrients, the trehalose reserve is slowly consumed, apparently serving as carbon and energy source for survival of the resting cells. When it becomes exhausted the cells rapidly die (Panek 1963; Lillie and Pringle 1980; Barton et al. 1982; McBride and Ensign 1987b). Therefore, it appears to be reasonable to view trehalose as a reserve both for survival during starvation and for the stimulation of germination and the induction of growth.

B. Possible Role of Trehalose as Stress Protectant

Evidence that trehalose is able to act as a stress protectant both in vitro and in vivo has been accumulating at a fast pace in recent years. After the first clear-cut report by Crowe et al. (1984), many papers dealing with in vitro protective effects of trehalose on isolated proteins and membranes have been published. The role of trehalose as stress protectant has been reviewed, e.g., by Van Laere (1989), Wiemken (1990), and Crowe et al. (1992). More recent work has shown impressive and very specific protective effects of trehalose during desiccation of biological structures by drying at ambient temperatures (Crowe et al. 1990; Roser 1991a; Colaço et al. 1992). Because of this remarkable property, trehalose has been used increasingly as cryoprotectant for freeze storage of a variety of intact cells and organisms (e.g., Bhandal et al. 1985; Anchordoguy et al. 1988; Coutinho et al. 1988; Honadel and Killian 1988; De Antoni et al. 1989) and more recently also for the active preservation in dried form at room temperature of

restriction enzymes (Colaço et al. 1992), antibodies (Blakeley et al. 1990), and a wide variety of food products (Roser 1991b).

The protective effect of trehalose is not well understood at the molecular level. Two hypotheses have been proposed. The first hypothesis (the water-replacement hypothesis) proposes that trehalose replaces water molecules that are hydrogen-bonded to the surface of biological macromolecules and that are essential for the maintenance of tertiary structure. Hydrogen bonds with the multiple hydroxyl groups of trehalose would result in a better stabilizing effect under adverse physical conditions, such as desiccation, heat, and freezing, compared to hydrogen bonds with water molecules (Clegg 1985; Saenger 1989; Otting et al. 1991). The crucial importance of direct interaction between trehalose and the macromolecules has been emphasized by Crowe et al. (1990) for desiccation resistance as opposed to freezing resistance. A second theory is based on the tendency of trehalose solutions to undergo glass rather than crystal formation upon dessication, which would result in the establishment of a physical state particularly protective for embedded macromolecules. The glass capsule around the macromolecules would freeze their native shape and, in this way, would also prevent any distortion of their structure during dehydration (Burke 1985; Slade and Levine 1988; Franks et al. 1991; Roser 1991b; Colaço et al. 1992; Levine and Slade 1992). This theory, however, fails to explain the protective effects of trehalose at temperatures much higher than the glass transition temperature. The main problem with both theories appears to be the superiority of trehalose in conferring stress protection compared to molecules with a very similar structure, such as glucose, maltose, and sucrose (Green and Angell 1989; Crowe et al. 1990; Colaço et al. 1992). However, as pointed out by Roser (1991b), trehalose possesses a number of physical properties, which together might give it a unique functionality, different from other sugars. These properties include a very high hydrophilicity, nonhygroscopic glass formation, very high chemical stability, nonreducing character, and absence of internal hydrogen bond formation, resulting in unusual flexibility of the disaccharide bond.

One of the main arguments used for a physiological role of trehalose as stress protectant in living organisms is its presence in very high amounts in a wide variety of biological structures able to withstand extreme stress conditions, such as desiccation and heat (Van Laere 1989; Wiemken 1990; Crowe et al. 1992). Well-known examples in fungi are survival forms like spores and sclerotia and also commercial baker's yeast which can contain up to 15–20% of its dry weight in trehalose and for which the culture conditions have been optimized in order to obtain such a high trehalose content. With respect to in vivo evidence for a role of trehalose as stress protectant in yeast, it has to be emphasized that all results obtained untill now for all stress conditions investigated have been of a correlative nature. However, the body of evidence accumulated in this way is quite impressive and strongly supports the notion that in yeast high trehalose levels directly protect cells against different types of stress, such as heat, freezing, and desiccation.

It has long been known that stationary-phase yeast cells are more stress-resistant than exponentially growing cells, possibly because of their high trehalose content (Schenberg-Frascino and Moustacchi 1972; Parry et al. 1976; Walton et al. 1979; Iida and Yahara 1984; Gadd et al. 1987; Plesset et al. 1987; Elliott and Futcher 1993). Yeast cells growing at high temperature are also known to contain a higher trehalose level (Grba et al. 1979). The laboratory of Wiemken (1990) has made use of sublethal heat treatment and subsequent cooling to increase and to reduce again the trehalose content of *S. cerevisiae* cells. They demonstrated a close correlation between trehalose content and resistance to heat shock (a few min at 50 °C) (Hottiger et al. 1987a). Similar results were reported for *Schizosaccharomyces pombe* (De Virgilio et al. 1990) and *Neurospora crassa* (Neves et al. 1991), using the same approach. Yeast strains with mutations causing directly or indirectly reduced activity of cAMP-dependent protein kinase (cAMP-PK) contain more trehalose and are more heat-resistant than wild-type strains, while the opposite is true for strains with mutations causing enhanced activity of cAMP-PK (Martegani et al. 1986; Shin et al. 1987; Cameron et al. 1988; Iida 1988; Hottiger et al. 1989; Panek et al. 1989). Recently, Attfield et al. (1992) demonstrated a correlation between the trehalose level and resistance to heat and freeze-thaw stress in a series of related yeast strains. Contrary to their claim, however, there is no reason to assume that the strains were different only in the trehalose level and not in other properties. The reason for the difference in the trehalose level was not

known. It is important to point out that the trehalose levels present in laboratory yeast strains, e.g., in stationary-phase cells or in cells given a sublethal heat treatment, are still only about 3–4% of the dry weight and, therefore, much lower than the 15–20% which can be present in commercial baker's yeast. Whereas it is difficult to see how a trehalose concentration of about 1–2 M in the cytoplasm of commercial baker's yeast cells would not have an effect on stress resistance, this is not so obvious for the concentrations present in yeast cells grown under normal conditions. McBride and Ensign (1987a) demonstrated a positive correlation between trehalose content and heat resistance in spores of *Streptomyces griseus*. In *Escherichia coli* the *otsA* and *otsB* genes encoding trehalose-6-phosphate synthase and phosphatase respectively are induced during transition into stationary phase and are involved in stationary-phase thermotolerance (Hengge-Aronis et al. 1991).

Several studies have shown a positive correlation between trehalose content and freeze tolerance of yeast (Oda et al. 1986; Gélinas et al. 1989; Hino et al. 1990). These studies were conducted in connection with the preparation of frozen dough. Yeast cells rapidly deplete their trehalose level upon contact with rich media containing fermentable sugars, and this is considered to be the major reason why baker's yeast rapidly loses its freeze tolerance during dough preparation (Oda et al. 1986). Special dough preparation conditions minimizing fermentation activity prior to freezing (e.g., rapid mixing at low temperature) have to be used in order to preserve the vitality and gassing power of baker's yeast for freeze storage of the dough (Merritt 1960; Kline and Sugihara 1968; Sugihara and Kline 1968). Also in *Phycomyces* and in *Neurospora* spores, a positive correlation between freezing resistance and trehalose content has been demonstrated (Van Laere 1989).

A positive correlation between trehalose content and resistance against osmotic stress was demonstrated in *S. cerevisiae* by Mackenzie et al. (1988). Significant in this respect is also that many microorganisms synthesize large amounts of trehalose upon osmotic shock (Mackay et al. 1984; Reed et al. 1986; Strøm et al. 1986; Breedveld et al. 1991; Zevenhuizen 1992). In *Escherichia coli* trehalose-6-phosphate synthase and phosphatase, encoded by the *otsA* and *otsB* genes, and also periplasmic trehalase, encoded by the *treA* gene, are strongly induced upon osmotic shock and mutants deficient in trehalose synthesis display an osmotically sensitive phenotype (Boos et al. 1987; Giæver et al. 1988; Gutierrez et al. 1989; Kaasen et al. 1992; McDougall et al. 1993). A positive correlation between trehalose content and desiccation resistance has been demonstrated in *S. cerevisiae* (Zikmanis et al. 1985, 1988; Gadd et al. 1987; Hottiger et al. 1987a) and in spores of *Phycomyces* (Van Laere 1986b), *Mucor* (Van Laere and Slegers 1987), and *Streptomyces* (Martin et al. 1986; McBride and Ensign 1987a). Desiccation of *S. cerevisiae* cells is known to trigger mobilization of glycogen and concomitant accumulation of trehalose (Payen 1949; Pollock and Holmstrom 1951; Marino et al. 1989). Other stress conditions, such as exposure to toxic chemicals (ethanol, copper sulfate, hydrogen peroxide) also induced trehalose accumulation, indicating that it may be part of a general stress response in yeast (Attfield 1987).

A major problem with nearly all studies showing a correlation between trehalose level and thermotolerance in yeast is that virtually all conditions and mutations used are known to affect also heat shock protein synthesis. The only exception was addition of canavanine, which was claimed to induce heat shock proteins without causing an increase in the trehalose level or thermotolerance (Hottiger et al. 1989). Subsequent more detailed studies, however, revealed that canavanine in fact also increased both the trehalose level and thermotolerance in *Schizosaccharomyces pombe* and *S. cerevisiae* cells, but only after a long lag phase. The increases in trehalose and thermotolerance were closely correlated (Hottiger et al. 1992). The role of heat shock proteins in thermotolerance and in particular the acquisition of thermotolerance is also unclear (Mager and Moradas Ferreira 1993). The only exceptions in this respect appear to be the yeast heat shock protein hsp104 and the heat shock-induced catalase T, encoded by the *CTT1* gene. Deletion of *CTT1* causes a reduction in thermotolerance, possibly because at higher temperatures damage due to oxidative stress is more pronounced than at normal temperatures (Wieser et al. 1991). A strain with a deletion of the *HSP104* gene (*hsp104*Δ) is very sensitive to a wide variety of stresses such as heat, freezing, and desiccation, and acquires thermotolerance more slowly than the corresponding wild-type strain (Sanchez and Lindquist 1990; Sanchez et al. 1992). The same *hsp104*Δ strain has been used by Winkler et al. (1991), who

reported that deletion of hsp104 did not prevent trehalose accumulation during sublethal heat treatment (40 min at 39°C), while, on the other hand, the cells did not acquire thermotolerance (heat shock at 50°C). However, they measured trehalose content and thermotolerance only at one time point. De Virgilio et al. (1991c) measured trehalose levels and resistance against heat stress (a few min at 50°C) of *hsp104Δ* cells incubated for longer times at a sublethal temperature of 40°C. They observed an increase in both trehalose and in heat resistance, confirming a possible role for trehalose as stress protectant. However, the *hsp104Δ* strain acquired thermotolerance more slowly than the corresponding wild-type strain, in agreement with the data of the Holzer group (Winkler et al. 1991) and the original report by Sanchez and Lindquist (1990). This indicates that there is more than one determinant for heat resistance in yeast, a conclusion which is not surprising. The importance of heat shock proteins might be related more to repair of heat and stress-induced damages (Yost and Lindquist 1991) rather than to physical protection against stress, as in the case of trehalose and other small molecules acting as stress protection metabolites like glycerol, mannitol, sucrose, etc. Induction of heat-resistance without any effect on the trehalose level was observed upon treatment of *S. cerevisiae* cells with a plant cytokinin, confirming that other factors exist which are able to confer stress resistance (Coote et al. 1992). In *S. pombe*, heatinduced accumulation of trehalose and thermotolerance are not affected by the presence of cycloheximide, supporting the idea that for acquired thermotolerance during sublethal heat treatment trehalose accumulation is more important than heat shock protein synthesis (De Virgilio et al. 1990). In *S. cerevisiae*, on the other hand, inhibition of protein synthesis reduces acquired thermotolerance, but since it also reduces trehalose accumulation, it remains unclear whether the absence of heat shock protein synthesis or the reduction in trehalose accumulation is responsible (Hall 1983; De Virgilio et al. 1991c; Coote et al. 1992). Some of the discrepancies between the conclusions on the protective effect of trehalose and heat shock proteins might be due to the fact that a certain concentration of trehalose is required before its protective effect can be observed, and second, to the fact that trehalose only prevents stress-induced damage while heat shock proteins might also be able to correct stress-induced damage. Gadd et al. (1987) provided evidence that a certain minimal intracellular trehalose concentration would be required to confer dehydration resistance in yeast. They also showed that it was impossible to increase the desiccation resistance of exponential-phase cells above 10% survival by addition of high external concentrations of trehalose, whereas in stationary-phase cells 100% survival was easily obtained. This supports the idea that trehalose is effective as a stress protectant only in the presence of one or more other factor(s) found in stationary-phase cells and not in exponential-phase cells. This conclusion is in agreement with recent data showing a general but not precise correlation between trehalose level and freeze-thaw tolerance (Lewis et al. 1993).

The recent cloning of the *S. cerevisiae* genes involved in trehalose metabolism permits more specific manipulation of the trehalose content without the many possible side effects caused by using cells of different growth phases, of mutants affected in pleiotropic control systems like cAMP-metabolism, or cells given treatments like heat shock, desiccation, or exposure to toxic chemicals. Recent results show that absence of glucose-induced trehalose mobilization in stationary-phase cells does not prevent glucose-induced loss of stress resistance. Therefore the initiation of fermentation seems to cause the disappearance of other factors which are required for stress resistance even in the presence of a high trehalose level (Van Dijck et al. 1995, added in proof).

C. Main Functions of Trehalose

Based on the data presently available, it appears safe to conclude that trehalose has a dual function, both as a storage carbohydrate and as a stress protection metabolite, in particular for survival under adverse, starvation conditions. Other factors, however, also contribute to stress protection and resistance, and in their absence trehalose might not be very effective. The rapid mobilization of trehalose during spore germination and growth induction in stationary-phase cells in nutrient media containing ample glucose appears to be explained best by trehalose mobilization being part of the developmental program initiated in the presence of nutrients in the culture medium. The trehalose reserve was essential for energy and car-

bon provision in the preceding developmental stage, and might be helpful in the present stage under conditions of limited nutrient supply.

F. Other Functions of Trehalose

Trehalose has also been suggested to function as a long-distance translocation carbohydrate in fungi (Cochrane 1958). Evidence for such a role has been reported for hyphae of *Serpula lacrimans* (Brownlee and Jennings 1981) and *Agaricus bisporus* (Hammond and Nichols 1976). Translocation of trehalose from the hyphae of mycorrhizal fungi into the cells of orchid seedlings has also been demonstrated (Smith 1967).

VI. Regulation of Trehalose Metabolism

A. Mobilization of Trehalose

Induction of growth in stationary-phase cells or induction of germination in spores and survival forms is invariably associated with rapid mobilization of the trehalose reserve. As noted before, this also occurs in media containing ample sugar, leading to questions about the true function of the rapid mobilization of trehalose.

1. Decompartmentation Versus Activation of Trehalase

Two mechanisms have been proposed as triggers for the mobilization of trehalose: In the case of fungi with an acid trehalase, decompartmentation of trehalose and trehalase was suggested to be responsible, while in the case of fungi with a neutral trehalase, activation of the enzyme by cAMP-dependent protein phosphorylation was proposed as trigger (Thevelein 1984c). The decompartmentation mechanism was mainly based on the separate localization of trehalose (in the cytoplasm) and the acid trehalase (outside the plasma membrane). Rapid activity changes of the acid trehalase during trehalose mobilization were never observed, and breakdown of a permeability barrier between the two was therefore suggested to be responsible for the initiation of trehalose mobilization (Thevelein 1984c). New results obtained with *N. crassa* and *S. pombe*, however, offer alternative explanations. Detailed studies of heat shock-induced trehalose accumulation and subsequent mobilization by lowering the temperature in *Neurospora* hyphae by the group of H. Terenzi strongly suggest the existence of a second trehalase whose activity apparently cannot be detected in crude extracts (H. Terenzi, pers. comm.). In contrast to earlier reports, neutral trehalase activity has now been reported for *S. pombe*. It is present in the cytosol of vegetative cells and (in lower quantity) in the cytosol of ascospores (De Virgilio et al. 1991b). Recently, glucose-induced activation of neutral trehalase has also been reported for derepressed vegetative cells of *S. pombe* (Carrillo et al. 1992). The activation could be mimicked by exogenous cAMP and by several treatments known to enhance the cAMP level in *S. cerevisiae*. In general, the characteristics of the activation were very similar to those reported for *S. cerevisiae*. As pointed out by Carrillo et al. (1992), this raises an intriguing question about the importance of glucose-induced cAMP signaling in the activation process, since the Ras proteins in *S. pombe* are well known not to act on adenylate cyclase, which is also strikingly different in many respects from the *S. cerevisiae* adenylate cyclase (see below). These new results raise the possibility that also in ascospores of *S. pombe*, activation of trehalase by cAMP-dependent protein phosphorylation is responsible for trehalose mobilization, rather than decompartmentation of trehalose and acid trehalase. Hence, it appears possible that in all cases of rapid trehalose mobilization activation of neutral trehalase is responsible, but that in some cases the activation of the enzyme has remained undetected because of its lability. In *Dictyostelium discoideum*, on the other hand, efforts to detect a neutral trehalase able to be activated by cAMP-dependent protein phosphorylation under the same conditions as in *S. cerevisiae* remained unsuccessful. The authors concluded that decompartmentation of trehalose and the vacuolar acid trehalase was the only plausible mechanism for triggering rapid trehalose mobilization during early spore germination (Gupta et al. 1987).

2. Activation of Trehalase by Phosphorylation

Involvement of phosphorylation by cAMP-dependent protein kinase for in vitro activation of neutral trehalase in *S. cerevisiae* is now well established (Uno et al. 1983; App and Holzer 1989). In

vivo changes in trehalase activity, such as nutrient-(Coutinho et al. 1992; P. Durnez and J. Thevelein, unpubl. results) and heat-induced activation of trehalase (De Virgilio et al. 1991a) and the drop in activity during the diauxic shift (François et al. 1987; Coutinho et al. 1992) all appear to be mediated by phosphorylation/dephosphorylation. The lability of the neutral trehalase during purification has hampered detailed studies of its activation by phosphorylation. Phosphorylation in vivo, for instance, has never been demonstrated directly. However, the availability of the neutral trehalase gene (*NTH1*) offers new approaches to obtain information on the molecular mechanism of the activation, such as the identity of the phosphorylated amino acid residue. Since the predicted amino acid sequence of the *NTH1* gene indicates only one consensus site for cAMP-dependent protein phosphorylation, activation of the enzyme in vivo might in all cases be due to phosphorylation of the same amino acid residue. Site-directed mutagenesis of this consensus sequence should be able to give a definite answer to this important question. If all increases in trehalase activity observed in vivo in *S. cerevisiae* are due to phosphorylation of this site, trehalase would prove to be an excellent marker for the activity of cAMP-dependent protein kinase. At the same time, it would raise a number of interesting questions concerning the regulation of cAMP-dependent protein kinase (see below).

3. Glucose-Induced Activation of Trehalase

Glucose-induced activation of trehalase in *S. cerevisiae* was shown to be preceded by a rapid transient increase in the cAMP level both in derepressed vegetative cells (van der Plaat 1974) and in ascospores (Thevelein 1984a). Such a glucose-induced rise in the cAMP level was also observed during the initiation of spore germination in *Phycomyces* (Van Mulders and Van Laere 1984), *Mucor* (Dewerchin and Van Laere 1984), and *Pilobolus* (Bourret 1986). François et al. (1984) showed that in a *S. cerevisiae* temperature-sensitive mutant in cAMP synthesis, no glucose-induced activation of trehalase was observed at the restrictive temperature. Based on this result, they concluded that the glucose-induced rise in the cAMP level was required for glucose-induced activation of trehalase. However, for such a conclusion, a strain that retains the wild-type basal cAMP level and specifically lacks the glucose-induced increase in cAMP is required. Such strains have become available, e.g., *cdc25* p*RAS2*$^{Ile[3]}$ (Van Aelst et al. 1990) and *lcr1* (Becher dos Passos et al. 1992; Vanhalewyn and Thevelein 1992), and they unexpectedly still show glucose-induced activation of trehalase (L. Van Aelst, unpubl. results). The solution for this problem might lie in the existence of an alternative cAMP-independent activation pathway of cAMP-dependent protein kinase (see below). The recent finding that glucose also activates trehalase in *S. pombe* (Carrillo et al. 1992) may provide further support for such an alternative activation pathway, since adenylate cyclase and also its control are significantly different in *S. pombe* as compared to *S. cerevisiae* (Fukui et al. 1986; Engelberg et al. 1990). The Ras protein of *S. pombe* does not act on adenylate cyclase and a glucose-induced increase in the cAMP level has not been reported. Hence, it is possible that also in *S. pombe* only a basal level of cAMP is required for glucose-induced activation of trehalase.

Different mechanisms have been proposed for the activation of cAMP synthesis by glucose: transient plasma membrane depolarization, transient intracellular acidification, and a specific G-protein-mediated signal transduction pathway (for review, see Thevelein 1988, 1991, 1992). Results obtained with mutants in the Cdc25-Ras-adenylate cyclase pathway have made the old hypotheses of transient plasma membrane depolarization and transient intracellular acidification, as well as increased energy supply, very unlikely (see also below). They have supported the notion that in yeast, glucose and related rapidly fermented sugars trigger a specific signal transduction pathway similar to hormone-induced signaling pathways in higher organisms. Yeast adenylate cyclase is regulated by the RAS proteins which are members of the G-protein class. They are active in the GTP-bound form and inactive in the GDP-bound form. Exchange of GDP for GTP on the RAS proteins is stimulated by the *CDC25* gene product. How glucose activates Cdc25 has not yet been elucidated. Activation of cAMP synthesis by glucose requires glucose phosphorylation and also a relatively high glucose concentration (K_m = 15–20 mM). In addition, the glucose-induced cAMP signal is observed only in glucose-derepressed wild-type cells and in exponential-phase glucose-grown cells of glucose-repression mutants, suggesting the possible occurrence of a glucose-repressible protein in the path-

way. An alternative explanation might be that adenylate cyclase itself is glucose-repressible and therefore able to react much faster to glucose addition in derepressed than in repressed cells. The resulting difference in activity would not be reflected in a different cAMP level because of the strong feedback-inhibition of cAMP-dependent protein kinase on cAMP synthesis (Nikawa et al. 1987). The recent finding that the *lcr1* mutant, which lacks the glucose-induced cAMP increase under all conditions, has an insertion in the promotor of the *CYR1* gene, encoding adenylate cyclase (Vanhalewyn and Thevelein 1992), is consistent with such an explanation. No correlation is observed between the level of sugar phosphates and the induction of the cAMP signal, making it unlikely that sugar phosphates act as triggers (for review, see Thevelein 1991). In vitro reconstitution of glucose-induced activation of adenylate cyclase has been claimed (Pardo et al. 1991) but the characteristics of the process do not fit well with those observed in vivo.

Recently, the yeast mutants *fdp1* and *byp1*, both of which have a growth problem on media containing fermentable sugar, were shown to be defective in glucose-induced activation of cAMP synthesis as well as in many other glucose-induced regulatory events. The cloning of *FPS1*, a suppressor of *fdp1*, and *MIG1*, a suppressor of *byp1*, appeared to support the specific requirement of both gene products for glucose-induced cAMP signaling, since the suppressors restored only the growth defect and not the signaling defects (Van Aelst et al. 1991; Hohmann et al. 1992). Subsequently, *fdp1* and *byp1* were shown to be allelic and deletion mutants in the gene, which was called *GGS1*, displayed the same glucose-induced growth and regulatory defects (Thevelein 1992; Van Aelst et al. 1993). The gene was also cloned as complementing the allelic *cif1* mutant (Gonzalez et al. 1992). Unexpectedly, as mentioned before, the *GGS1* gene was also cloned independently as encoding a subunit of trehalose-6-phosphate synthase/phosphatase and called *TPS1* (Bell et al. 1992; Vuorio et al. 1992). This has raised the intriguing question of how trehalose synthesis or at least the *GGS1/TPS1* subunit could be involved in the control of initial glucose metabolism (see below). Recent work also showed that additional deletion of *HXK2*, the gene encoding the most active hexokinase, in a *ggs1/tps1*Δ mutant restored growth on glucose and also glucose-induced signaling, but not the trehalose level (Hohmann et al. 1993). This points to sugar phosphorylation as the cause of the growth defect on fermentable sugars. It also indicates that the *GGS1/TPS1* gene product might not be involved in glucose-induced signaling, although this should be viewed with caution since deletion of hexokinase 2 does not only affect sugar phosphorylation but also abolishes glucose repression (Ma and Botstein 1986; Rose et al. 1991), and therefore possibly other regulatory connections. Reduction of sugar influx into glycolysis by alternative means, e.g., reduction of glucose transport by deletion of sugar carrier genes, might be able to give an answer to the question of *GGS1/TPS1* involvement in glucose-induced signaling and glucose-induced activation of the Cdc25-Ras-adenylate cyclase pathway in particular. In addition, a possible involvement of the *GGS1/TPS1* homologous parts of the *TSL1*, *TPS2*, and *TPS3* genes in glucose-induced signaling cannot be excluded. The transient character of the glucose-induced cAMP increase in derepressed yeast cells appears to be due to feedback inhibition of cAMP synthesis by cAMP-PK, since the transient character gradually decreases when cAMP-PK activity is lowered. In mutants with strongly reduced activity of cAMP-PK, there is a continuous glucose-induced increase in the cAMP level, which is clearly inconsistent with transient membrane depolarization or intracellular acidification as triggering mechanism for glucose-induced activation of cAMP synthesis (Mbonyi et al. 1990).

4. Fermentable Growth Medium-Induced Activation of Trehalase

Addition of only glucose to stationary-phase yeast cells or to yeast ascospores causes only a transient mobilization of trehalose followed by resynthesis. The trehalose pattern closely correlates with the proliferation behavior, since stationary-phase cells rapidly arrest again as G0 cells when they are starved for an essential nutrient, such as nitrogen, phosphate, or sulfate. Ascospores also need a full medium for complete germination. The course of trehalase activity closely follows the changes in the trehalose level. When only glucose is given, both trehalase activation and trehalose mobilization are transient. When a full medium with a fermentable carbon source is given, trehalase remains activated for a much longer time and trehalose is completely degraded (for review, see Thevelein 1984c). When only glucose is given, or

when in a glucose-containing medium an essential nutrient is omitted, the cells will arrest their proliferation as G0 unbudded cells and accumulate a large amount of trehalose. Subsequent addition of the lacking nutrient, e.g., nitrogen, phosphate, or sulfate, in the presence of glucose triggers rapid activation of trehalase and mobilization of trehalose. Growth stimulation by addition of a good nitrogen source to cells growing on a poor nitrogen source has the same effect (Hirimburegama et al. 1992). Since activation by other nutrients always requires the presence of a fermentable carbon source, we suggest calling this pathway the fermentable growth medium-induced pathway. Detailed investigation of cAMP levels and the use of mutants in cAMP metabolism clearly demonstrated that activation of trehalase in glucose-repressed cells by other nutrients, such as nitrogen sources, phosphate, or sulfate is not mediated by cAMP (Thevelein and Beullens 1985; Hirimburegama et al. 1992; P. Durnez, unpubl. results). In spite of this, nitrogen source-induced activation of trehalase appears to be mediated by phosphorylation, and requires the activity of the catalytic subunits of cAMP-dependent protein kinase, while presence of the regulatory subunit reduces the activation (P. Durnez, unpubl. results). We have suggested that a novel signaling pathway induced by a complete fermentable growth medium converges with the cAMP pathway at the level of the free catalytic subunits, and causes their activation in a cAMP-independent way (Thevelein 1991). Further indirect support for this alternative mechanism came from the observation that glucose-induced activation of cAMP synthesis was observed only in glucose-derepressed yeast cells and not in glucose-repressed cells. The requirement of glucose for activation of trehalase by other essential nutrients can therefore not be attributed to its activating effect on cAMP synthesis through the Cdc25-Ras-adenylate cyclase pathway (Thevelein 1991). Studies on nutrient-induced transcriptional control of several genes, e.g., *CTT1* and *GGS1/TPS1*, have revealed a second system, apparently mediated by the same fermentable-growth-medium induced pathway (see below).

5. Trehalase Activity During the Cell Cycle

Küenzi and Fiechter (1969) reported a periodic fluctuation in trehalase activity in self-synchronized chemostat cultures of *S. cerevisiae*, and attributed this to periodic synthesis of trehalase during the cell cycle. However, as mentioned before, this self-synchronization phenomenon is the result of a synchronous exit of the whole population from the stationary G0 phase into the cell cycle followed by a new stay in the G0 phase, new entry into the cell cycle, etc. rather than a continuously synchronized cell cycle itself. The fluctuations in trehalase activity are therefore associated with the exit from G0 rather than with the transition from one cell cycle phase into another one. Consistent with this conclusion is the absence of any fluctuation in trehalase activity during the cell cycle in synchronous cultures obtained by fractionation of exponential-phase cultures according to cell size by means of elutriation centrifugation (de Koning et al. 1991, in contrast to earlier reports by van Doorn et al. 1988a,b).

B. Synthesis of Trehalose

In *S. cerevisiae* there is a strong correlation between growth rate and trehalose content. Yeast cells growing on fermentable sugars like glucose and fructose have the highest growth rate and the lowest trehalose level, stationary-phase cells have the highest trehalose level while cells growing on nonfermentable carbon sources have a low growth rate and an intermediate trehalose level (Thevelein 1984c). Yeast cells incubated with a fermentable carbon source like glucose and starved for another essential nutrient like nitrogen, accumulate large amounts of trehalose (Küenzi and Fiechter 1972; Lillie and Pringle 1980). In the absence of external sugar, conversion of glycogen into trehalose can be induced under specific conditions: oxygenation (Callaerts et al. 1993), desiccation (Payen 1949; Marino et al. 1989), and formation of ascospores (Roth 1970).

1. Regulation of Trehalose-6-Phosphate Synthase and Phosphatase at the Posttranslational Level

One possible explanation for the differences in the trehalose level observed during the growth cycle is control of the enzymes of trehalose metabolism at the posttranslational level: activation of trehalase and inactivation of trehalose-6-phosphate synthase by cAMP-dependent protein phosphorylation. Whereas control of trehalase by cAMP-dependent protein phosphorylation is well

established, there has been controversy whether trehalose-6-phosphate synthase in *S. cerevisiae* is regulated by phosphorylation. The group of Panek claimed that the synthase was reversibly inactivated by phosphorylation (Panek et al. 1987). The assay method used has been criticized, however, by Vandercammen et al. (1989), who also could not find evidence for inactivation through phosphorylation either in vivo or in vitro. Initially, Vandercammen et al. (1989) also contradicted the observation by the Panek group (Panek et al. 1987) that yeast mutants with increased or decreased activity of cAMP-PK had, respectively, lower and higher activity of trehalose-6-phosphate synthase. Subsequently, however, they reported that mutants with increased cAMP-PK activity and a strain able to take up cAMP from the medium and incubated with cAMP had strongly decreased trehalose-6-phosphate synthase and phosphatase activity, while a mutant with lower cAMP-PK activity might retain somewhat higher activity during growth on glucose (François et al. 1991).

Panek et al. (1987) also reported glucose-induced inactivation of trehalose-6-phosphate synthase. This effect was confirmed and studied in more detail by François et al. (1991), who showed that glucose-induced inactivation was dependent on the presence of a nitrogen source in a way similar to that previously reported for activation of trehalase (Thevelein and Beullens 1985). Probably, glucose-induced activation of trehalose-6-phosphate synthase is triggered by the same fermentable growth medium-induced pathway which appears to activate the catalytic subunits of cAMP-dependent protein kinase in a cAMP-independent way (Thevelein 1991). This pathway might play a crucial role in a wide range of phenomena occurring during the diauxic shift in yeast cultures or triggered by nitrogen starvation on glucose-containing media. Based on reversibility studies and experiments using cycloheximide, François et al. (1991) concluded that glucose-induced inactivation of trehalose-6-phosphate synthase was probably due to proteolytic inactivation, and that the effect of high protein kinase activity was probably exerted at the transcriptional level rather than by posttranslational modification, as suggested by Panek et al. (1987). The latter conclusion fits with recent data showing that expression of the *GGS1/TPS1* gene is repressed in mutants with enhanced activity of cAMP-PK and derepressed in mutants with reduced activity of cAMP-PK (Winderickx et al. unpubl. results). As a result of the criticisms by Vandercammen et al. (1989), the group of Panek (1990) reinvestigated trehalose-6-phosphate synthase activity using different assay methods, and they confirmed the validity of the assay, the differences in activity in the Ras-cAMP pathway mutants, the increase in activity upon entry into stationary phase, and the subsequent inactivation by re-addition of glucose to the cells. On the other hand, the previously reported experiments on in vitro activation of trehalose-6-phosphate synthase by dephosphorylation and in vitro inhibition of the enzyme by phosphorylation were not repeated, or at least their reproducibility was not confirmed. Because of the sensitivity of the complex to proteolysis and the activation of synthase activity by partial proteolysis (Londesborough and Vuorio 1991), results obtained in vitro with this enzyme have to be regarded with much caution. It appears safe to conclude at present that most of the differences observed in trehalose-6-phosphate synthase activity are due to regulation at the transcriptional level. The rapid change in activity observed during glucose-induced inactivation is probably due to proteolysis, although the evidence in this respect is still preliminary and also does not exclude a mechanism involving phosphorylation followed by proteolysis. In general, involvement of phosphorylation in regulation of the enzyme remains uncertain.

In *Candida utilis* two UDPG-utilizing trehalose-6-phosphate synthase enzymes have been reported. Evidence was presented for in vitro activation of the first enzyme by partial proteolysis and in vitro regulation of the second enzyme by phosphorylation/dephosphorylation. As in *S. cerevisiae*, evidence for the latter has to be considered preliminary (Vicente-Soler et al. 1989, 1991). Addition of glucose to stationary-phase cells of *Candida utilis* also caused a rapid partial loss of total trehalose-6-phosphate synthase activity. Which one of the two enzymes was affected has not been investigated.

2. Regulation of Trehalose-6-Phosphate Synthase and Phosphatase at the Transcriptional Level

As mentioned above, the trehalose-6-phosphate synthase/phosphatase complex in *S. cerevisiae* is encoded by the *GGS1/TPS1*, *TSL1*, *TPS2*, and possibly also the *TPS3* gene. Only the regulation

of *GGS1/TPS1* expression has been studied in detail (Winderickx et al., unpubl. results). *GGS1/TPS1* expression strongly increases during the diauxic shift (i.e., transition from growth on glucose to growth on ethanol) and during entrance into the G0 phase by starvation of cells for nitrogen on a glucose-containing medium. In addition, *GGS1/TPS1* expression is strongly enhanced in mutants with low activity of cAMP-dependent protein kinase and strongly diminished in mutants with elevated activity of cAMP-dependent protein kinase. In all strains lacking the regulatory subunit of cAMP-dependent protein kinase, *GGS1/TPS1* expression was still enhanced in stationary-phase glucose-grown cells, indicating cAMP-independent nutrient control. Interestingly, on glucose-containing medium, *GGS1/TPS1* was derepressed by heat shock. We have suggested that *GGS1/TPS1* is under repression by a fermentable growth medium-induced pathway that is mediated by the catalytic subunits of cAMP-PK and counteracted by heat shock (Winderickx et al. unpublished results). For all these conditions, including nutrient availability, heat shock, and the mutants in the cAMP pathway, there is a strong correlation between *GGS1/TPS1* expression and previously reported data for trehalose content (Lillie and Pringle 1980; Toda et al. 1985; Hottiger et al. 1987b).

A control of gene expression very similar to that found for *GGS1/TPS1* has been reported for a set of other genes including *CTT1*, *UBI4*, *ADH2*, *SSA3*, *HSP12*, and *CYC7* (Tanaka et al. 1988; Bissinger et al. 1989; Cherry et al. 1989; Werner-Washburne et al. 1989; Boorstein and Craig 1990; Praekelt and Meacock 1990; Pillar and Bradshaw 1991; Marchler et al. 1992). In the promoter of the *DDR2* gene, another gene derepressed by heat shock on a fermentable growth medium, an element with the nucleotide sequence CCCCT has been identified as crucial for heat shock-induced derepression and the same or similar sequences have been found in the promoters of several members of the *CTT1* set of genes (Kobayashi and McEntee 1993). The *GGS1/TPS1* promoter contains several CCCCT sequences, and one of them is located in a consensus sequence previously identified in the *CTT1* promoter as inferring induction by heat shock as well as by nitrogen starvation and low activity of the Ras-cAMP pathway (Marchler et al. 1992; Winderickx et al. unpubl. results). Hence, the C_2T promoter sequence might be responsible for the coordinate regulation of the whole *GGS1/TPS1-CTT1* set of genes in response to changes in the environmental conditions.

Many other systems might also be under control of the fermentable growth medium-induced pathway. Glycogen, for instance, is well known to accumulate not only upon glucose exhaustion during the diauxic shift but also during starvation for nitrogen, sulfur, or phosphorus on a glucose-containing medium (Rothman-Denes and Cabib 1970; Lillie and Pringle 1980; Becker et al. 1982). In addition, as has been described for trehalose (Thevelein 1984c), a negative correlation exists between growth rate and glycogen content (Küenzi and Fiechter 1972; Costa-Carvalho et al. 1983). Two genes are present in *S. cerevisiae* encoding glycogen synthase, a constitutively expressed *GSY1* gene responsible for about 15% of the activity and a second gene *GSY2*, the expression of which increases strongly when cells enter stationary phase (Farkas et al. 1990, 1991). Interestingly, the *GSY2* promoter contains two CCCCT sequences, while none is present in the promoter sequence reported for *GSY1*. Hence, the *GSY2* gene might well be another example of a gene regulated by the fermentable growth medium-induced pathway.

3. Trehalose Accumulation During Sublethal Heat Treatment

The mechanism of trehalose accumulation by sublethal heat treatment is not well understood. The activity of trehalose-6-phosphate synthase increases during heat treatment in *S. pombe* (De Virgilio et al. 1990) as well as in *S. cerevisiae* (Hottiger et al. 1987b; Neves and François 1992). The latter might be due to the known heat shock induction of the *GGS1/TPS1* gene (Bell et al. 1992; Winderickx et al., unpubl. results). Heat shock also induces the *TPS2* gene and a concomitant increase in trehalose-6-phosphate phosphatase activity (De Virgilio et al. 1993). The increase in trehalose-6-phosphate synthase activity during sublethal heat treatment, however, is relatively slow (Hottiger et al. 1987b; Neves and François 1992). When measured after a heat treatment of only 20 min, no or only a small change in activity is observed in spite of a large increase in trehalose content over such a period (Winkler et al. 1991). This explains a seeming contradiction between the results of these groups. In addition, in *S. cerevisiae* (De Virgilio et al. 1991c; Winkler et al. 1991;

Neves and François 1992), as well as in *S. pombe* (De Virgilio et al. 1990) and *N. crassa* (Neves et al. 1991), heat-induced accumulation of trehalose is largely insensitive to cycloheximide. On the other hand, a strong inhibition was reported with the RNA synthesis inhibitors, acridine orange and ethidium bromide (Attfield 1987). These drugs, however, are known to affect other processes at similar concentrations (e.g., cAMP synthesis: Thevelein and Beullens 1985). Although trehalose-6-phosphate synthase activity still increases in the presence of cycloheximide in *S. pombe* (De Virgilio et al. 1990) and to some extent in *S. cerevisiae* (De Virgilio et al. 1991c), apparently due to posttranslational regulation, the rapid kinetics of trehalose accumulation, compared to the changes in enzyme activity, are more in agreement with a stimulation at the level of substrate availability. According to Winkler et al. (1991), the major reason for trehalose accumulation during sublethal heat treatment is the enhanced level of the substrates of trehalose-6-phosphate synthase, glucose-6-phosphate, and UDP-glucose. Their increase might be due to a differential effect of temperature on glucose influx and glycolytic capacity causing metabolites upstream in glycolysis to accumulate. Recently, this explanation has been challenged by Neves and François (1992), who reported a decrease rather than an increase in glucose-6-phosphate levels during sublethal heat treatment. The level of UDP-glucose, however, also showed a heat-induced increase in their measurements. The authors claimed that temperature-induced changes in the kinetic characteristics of trehalase and trehalose-6-phosphate synthase/phosphatase, strongly favoring trehalose synthesis, were responsible for heat-induced trehalose accumulation. Future work should at least be able to resolve the inconsistency in the glucose-6-phosphate measurements. Sublethal heat treatment also causes increased activity of trehalase which is partly due to enhanced expression of the protein and partly to artificial activation during the rapid cooling of the cells after the heat treatment (De Virgilio et al. 1991a). The latter was shown previously for heat-induced activation of trehalase in yeast ascospores (Thevelein 1984b).

4. An Alternative Maltose-Induced Pathway for Trehalose Synthesis

The group of Panek has reported results indicating a specific connection between maltose metabolism and trehalose accumulation. They suggested the existence of an alternative maltose-induced pathway for trehalose synthesis. This was based mainly on two arguments: (i) the mutants *ssa1*, *fdp1*, and *glc1* are unable to accumulate trehalose under whatever condition except when incubated with maltose and provided they contain a *MAL* gene, and (ii) all strains (wild-type, *ssa1*, *fdp1*, or *glc1*) with a constitutive *MAL* allele (*MAL*^{c}) accumulate trehalose under conditions where it is normally not accumulated, i.e., during growth on glucose medium (Panek et al. 1980; Operti et al. 1982; Padrao et al. 1982; Paschoalin et al. 1986). The group of Panek also identified an ADPG-dependent trehalose-6-phosphate synthase thought to be responsible for specific maltose-induced trehalose synthesis (Paschoalin et al. 1989). Further work is required to find out whether the gene encoding this enzyme is an ADPG-using functional homologue of *GGS1/TPS1* which forms a trehalose-6-phosphate synthase/phosphatase complex together with the *TSL1*, *TPS2*, and possibly *TPS3* gene products, or whether it encodes (part of) an entirely different trehalose synthase complex or pathway.

VII. Trehalose Metabolism and Control of Growth, Cell Cycle Progression, and Sporulation

As mentioned above, there is a strong correlation between trehalose level and growth rate in yeast as well as in many other fungi. In *S. cerevisiae*, exponential-phase cells growing on glucose have the highest growth rate and a very low trehalose level. G0 cells which are in stationary phase have the highest trehalose level, while cells growing exponentially on glycerol display intermediate values for both growth rate and trehalose level. Another factor besides nutrient availability known to control cell cycle progression and G0 entrance in yeast, is the RAS-adenylate cyclase pathway (for reviews see Matsumoto et al. 1985; Broach and Deschenes 1990; Thevelein 1992). Depletion of cAMP causes G1 arrest and G0 entrance, while an overactive cAMP-PK causes failure to arrest properly in G1 and to enter G0. Because nutrient starvation and cAMP depletion cause arrest at the same site of the start point in the G1 phase of the cell cycle, it has been proposed that cAMP acts as a second messenger for

control of cell cycle progression by nutrient availability (Jacquet and Camonis 1985; Dumont et al. 1989; Engelberg et al. 1989; Gibbs and Marshall 1989; Malone 1990). This idea also fits with the transcriptional control of the *GGS1/TPS1-CTT1* set of genes, whose expression is controlled by nutrient availability and cAMP-PK activity in a consistent manner (see below). However, no convincing evidence could be obtained for nutrient-induced activation of cAMP synthesis, except in the case of glucose addition to derepressed yeast cells (see below). In addition to nutrient-induced activation of trehalase, nutrient-induced control of the *CTT1* (Belazzi et al. 1991) and *GGS1/TPS1* gene (Winderickx et al., unpubl. results) is still observed in yeast strains lacking the regulatory subunit of cAMP-PK, providing further evidence that cAMP is not the mediator of the nutrient effect. The requirement of cAMP-PK for these nutrient-induced controls, and the fact that cAMP cannot be the mediator, led us to propose a mechanism in which a novel pathway activates the free catalytic subunits of cAMP-dependent protein kinase in a cAMP-independent way (Thevelein 1991). We suggest calling this pathway the fermentable growth medium-induced pathway. It is only triggered by a complete growth medium containing all essential nutrients and a fermentable carbon source. Because of the close correlation between the activity of this pathway and progression through the cell cycle or entrance into G0, it is tempting to speculate that the same pathway also controls, in a antagonistic manner to the *GGS1/TPS1-CTT1* set of genes, the synthesis of the G1 cyclins, at least in a medium containing a fermentable carbon source (Thevelein 1991). The G1 cyclins stimulate the *CDC28*-encoded protein kinase which triggers progression over the mating pheromone site of the start point (Reed 1992).

Since the fermentable growth medium-induced pathway requires glucose or another fermentable carbon source, the question arises again how the glucose is sensed by the cells. This might be through the same sensing mechanism (e.g., the hypothetical general glucose sensor) as for other glucose-induced signaling phenomena or through a separate mechanism. One argument for a common mechanism is that the level of glucose required for nitrogen-induced activation of trehalase is very similar to the level required for other glucose-induced signaling phenomena (Hirimburegama et al. 1992). Further work is required, however, to unravel the details of the glucose-sensing mechanism.

We have recently reported that diploid homozygotic *ggs1 ips1* and *fdp1* strains are unable to sporulate, while homozygotic *byp1* diploids show reduced sporulation (Van Aelst et al. 1993). Homozygotic diploids of *ssn1*, another allele of *GGS1/TPS1* (Charlab et al. 1985), were reported to show both strongly reduced sporulation and spore viability (Panek and Bernardes 1983). During yeast sporulation, intensive trehalose synthesis occurs and mature ascospores contain exclusively trehalose as reserve carbohydrate (Roth 1970; Barton et al. 1982; Thevelein et al. 1982). However, it is difficult to understand how trehalose synthesis itself would be required for the sporulation process. In addition, sporulation occurs in the absence of glucose, so that the sporulation defect of *ggs1/ips1* mutants cannot be due to glucose-induced deregulation.

VIII. Trehalose Metabolism and the Control of Initial Glucose Metabolism

The recent discovery that the *GGS1/TPS1*-encoded subunit of trehalose-6-phosphate synthase is required for proper control of initial glucose metabolism presents a puzzling problem. Previously, trehalose had only been considered as a reserve substance or stress protection metabolite. This finding appeared to point to another function for trehalose synthesis.

The inability of a mutant deficient in trehalose synthesis to grow on glucose is puzzling because of several reasons. (i) Yeast strains with elevated activity of cAMP-PK also lack trehalose and they grow without problems on glucose and other rapidly fermented sugars (Toda et al. 1985; Hottiger et al. 1989). (ii) Strains with elevated activity of cAMP-PK also lack glycogen (Toda et al. 1985) and other specific glycogen-deficient strains also do not have a growth problem on glucose (Farkas et al. 1991). (iii) When glucose is given to derepressed yeast cells, they do not show a net synthesis of trehalose but rather a rapid net mobilization of trehalose triggered by activation of trehalase, while trehalose synthesis is actually shut off by inactivation of trehalose-6-phosphate synthase. (iv) Furthermore, yeast cells growing exponentially on glucose do not synthesize trehalose and the activity of trehalose-6-phos-

phate synthase is very low under such conditions. There is also no futile cycle of trehalose synthesis and breakdown, since *tps2*Δ mutants which are deficient in trehalose-6-phosphate phosphatase accumulate trehalose-6-phosphate only under conditions where wild-type strains accumulate trehalose (De Virgilio et al. 1993).

We have proposed a model that explains how trehalose synthesis could be important for the initiation of glycolysis in cells growing on a gluconeogenic carbon source or on slowly fermented sugars like galactose and maltose. We suggested that the rapid influx and phosphorylation of glucose could deplete intracellular phosphate which is required further downstream in glycolysis at the level of glyceraldehyde-3-phosphate dehydrogenase. Rapid synthesis of trehalose would release free inorganic phosphate which would become available again to maintain the flux through the second part of glycolysis (Hohmann et al. 1993). The importance of trehalose synthesis in phosphate homeostasis would be restricted to the early transition period from the derepressed to the repressed state, after which other regulatory mechanisms would take over and fully adapt metabolism to fermentative conditions. Measurement of trehalose synthesis in a strain lacking trehalase or trehalose-6-phosphate synthesis in a strain lacking trehalose-6-phosphate phosphatase immediately after glucose addition is required to make a precise quantitative assessment of the flux into trehalose under these conditions. This will allow an estimation of the possible contribution of trehalose synthesis to phosphate recovery. Subsequent reduction of *GGS1/TPS1* expression should allow an assessment of the physiological relevance of rapid trehalose synthesis for phosphate recovery.

Recent data indicate that the *GGS1/TPS1* gene product is not only required to make the transition from the derepressed state to the repressed state but also for exponential growth on glucose, since a *ggs1/tps1*Δ p*GGS1/TPS1* strain that is growing on a glucose-containing medium also loses the capacity to grow on glucose when it loses the *GGS1/TPS1*-containing plasmid (S. Hohmann and J.M. Thevelein, unpubl. results). Another remarkable finding, which also argues against a flux problem, is the extreme sensitivity of *ggs1/tps1* mutants for glucose. Addition of less than 1 mM glucose to *ggs1/tps1* cells growing on 100 mM galactose causes already a significant growth inhibition (S. Hohmann and J.M. Thevelein, unpubl. results). These data seem to point to aberrant control of glucose influx or phosphorylation in the *ggs1/tps1* mutants. Suppression of the glucose growth problem by deletion of the *hxk2* gene encoding hexokinase PII, the most active glucose phosphorylating enzyme, is consistent with this conclusion (Hohmann et al. 1993). We have previously proposed a model in which transport-associated phosphorylation of glucose mediated by interaction of glucose carriers and sugar kinase enzymes would be responsible for the restriction of glucose influx into glycolysis. Because of the deficiency of *ggs1/tps1* mutants in many glucose-induced regulatory phenomena, we suggested the existence of a glucose-sensing complex composed of a glucose carrier, a sugar kinase, and in addition, the *GGS1/TPS1* gene product (and possibly other components). This complex was suggested to be responsible for control, and in particular restriction of glucose influx as well as activation of several glucose-induced signal transduction pathways (Thevelein 1992). Detailed measurements of glucose transport in *ggs1/tps1*Δ strains, however, indicate that there is no change in the kinetic characteristics, either V_{max} or K_m, as compared to wild-type cells (J. Ramos, S. Hohmann, and J.M. Thevelein, unpubl. results). Hence, the total influx of glucose into the cells does not appear to be changed by the *ggs1/tps1*Δ mutation. In wild-type *S. cerevisiae* cells, free intracellular glucose is very low. The rapid appearance of free intracellular glucose in the *ggs1/tps1* mutants (Van Aelst et al. 1993), including the *ggs1/tps1*Δ *hxk2*Δ strain, that grows without problems on glucose (Hohmann et al. 1993), is consistent with the idea that transport-associated phosphorylation of glucose is involved in the control of glucose influx into glycolysis, e.g., by restriction of hexokinase activity, and that the *GGS1/TPS1* gene product is required for its proper functioning. Deletion of *HXK2* in the *ggs1/tps1*Δ strain also restored the glucose-induced regulatory phenomena, indicating that the *GGS1/TPS1* gene product is dispensable for glucose-induced signaling (Hohmann et al. 1993). Since deletion of *HXK2* also affects glucose-induced regulatory phenomena, as it abolishes glucose repression, this conclusion should be viewed with caution. Reduction of the rate of sugar transport by deletion of sugar carrier genes might represent an alternative way to restore growth in the *ggs1/tps1* mutants and to evaluate the possible involvement of *GGS1/TPS1* in glucose-induced signaling.

In conclusion, it is now clear that the *GGS1/TPS1* encoded subunit of the trehalose-6-phosphate synthase/phosphatase complex is involved in the control of glucose influx into glycolysis in *S. cerevisiae*, most probably through a specific regulatory mechanism independent of trehalose synthesis.

The remarkable relationship between trehalose metabolism and the control of initial glucose metabolism is not unique to *S. cerevisiae*. Recently, *GGS1* homologues have been cloned in *Kluyveromyces lactis* (K. Luyten, J.M. Thevelein, and S. Hohmann, unpubl. results) and *S. pombe* (Blazquez et al. 1992), and their deletion also results in a pleiotropic phenotype as in *S. cerevisiae*. Interestingly, involvement of trehalose metabolism in glucose transport has also been proposed for glucose absorption in mammalian intestine (Sacktor 1968; Sacktor and Berger 1969), an idea which has apparently been dropped in the meantime for several reasons (Semenza 1981).

IX. Conclusions

Trehalose is a disaccharide widely present in fungi, and probably serves both as a storage carbohydrate and, when present in high concentration, also as a stress protectant. The recent cloning of the genes of trehalose metabolism in yeast will allow more specific manipulation of the trehalose content and its mobilization and accumulation and should, therefore, allow more definite conclusions in the near future concerning the precise role of trehalose as storage carbohydrate and as stress protectant.

The trehalose content of fungal cells shows dramatic changes in both the growth cycle and the life cycle. Nutrient-induced trehalose mobilization has been studied in detail and has led to the discovery of a glucose-induced cAMP-dependent protein phosphorylation cascade triggering, or at least stimulating, the initiation of growth in resting cells and spores. Induction of trehalose mobilization by activation of neutral trehalase through protein phosphorylation might be more wide spread among fungi than initially thought. The availability of the neutral trehalase gene of yeast should allow the cloning of other fungal trehalase genes and might confirm this idea. The hypothesis of decompartmentation between trehalose and acid trehalase as a trigger for trehalose mobilization, on the other hand, has received only little attention.

Detailed studies on the glucose-sensing mechanism responsible for activation of cAMP synthesis in yeast have led to the remarkable discovery that a subunit of the trehalose-6-phosphate synthase/phosphatase complex is critically involved in the control of glucose influx. This finding has already been extended to other species. Further studies along these lines will probably reveal a novel regulatory mechanism, controlling initial glucose metabolism. The pleiotropic effects of mutations in the protein suggest that it probably also has a more general regulatory function. Novel mechanisms also appear to be involved in the control by other nutrients of trehalase activity and also in the expression of trehalose-6-phosphate synthase activity at the transcriptional level.

The close correlation between growth rate and trehalose content in fungi might offer an unusual but very promising avenue to elucidate the mechanisms involved in nutrient-induced growth control, and this clearly offers very exciting perspectives for future research in this field.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 02/00216

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01R33/46

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, INSPEC, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J.C. WALLACE ET AL.: "Classification of 1H Spectra of Biopsies ..." MAGNETIC RESONANCE IN MEDICINE, vol. 38, 1997, pages 569-576, XP000955430	1,5,19, 37
A	the whole document	2-4
A	R.L. SOMORJAI ET AL.: "Computerized Consensus Diagnosis ..." MAGNETIC RESONANCE IN MEDICINE, vol. 33, 1995, pages 257-263, XP000955431 the whole document	1,19,37

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[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 June 2002	26/06/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Volmer, W

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 02/00216

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	P. HAHN ET AL.: "The Classification of Benign and Malignant Human Prostate Tissue..." CANCER RESEARCH, vol. 57, 1997, pages 3398-3401, XP000982466 the whole document	1,19,37

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

PCT

WRITTEN OPINION

(PCT Rule 66)

To:

RICHES, McKENZIE & HERBERT LLP
2 Bloor Street East
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Toronto, Ontario M4W 3J5
CANADA

Date of mailing *(day/month/year)*	25/10/2002
Applicant's or agent's file reference: P12202	REPLY DUE within 1 / 00 months/days from the above date of mailing

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/CA 02/ 00216	21/02/2002	21/02/2001

International Patent Classification (IPC) or both national classification and IPC

G01R33/46

Applicant

NATIONAL RESEARCH COUNCIL OF CANADA

1. This written opinion is the first drawn up by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

- I [X] Basis of the opinion
- II [ ] Priority
- III [ ] Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV [ ] Lack of unity of invention
- V [X] Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI [ ] Certain documents cited
- VII [ ] Certain defects in the international application
- VIII [ ] Certain observations on the international application

3. The applicant is hereby **invited to reply** to this opinion.

When? See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also For an additional opportunity to submit amendments, see Rule 66.4.
For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4*bis*.
For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 21/06/2003

Name and mailing address of the IPEA/	Authorized officer / Examiner
European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465	Formalities officer (incl. extension of time limits) Tel. (+49-89) 2399 2828

Form PCT/IPEA/408 (cover sheet) (march 2002)

I. Basis of the opinion

1. The basis of this written opinion is the application as originally filed.

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability

1. In light of the documents cited in the international search report, it is considered that the invention as defined in at least some of the claims does not appear to meet the criteria mentioned in Article 33(1) PCT, i.e. does not appear to be novel and/or to involve an inventive step (see international search report, in particular the documents cited X and/or Y and corresponding claims references).

2. If amendments are filed, the applicant should comply with the requirements of Rule 66.8 PCT and indicate the basis of the amendments in the documents of the application as originally filed (Article 34 (2) (b) PCT) otherwise these amendments may not be taken into consideration for the establishment of the international preliminary examination report. The attention of the applicant is drawn to the fact that if the application contains an unnecessary plurality of independent claims, no examination of any of the claims will be carried out.

NB: Should the applicant decide to request detailed substantive examination, then an international preliminary examination report will normally be established directly. Exceptionally the examiner may draw up a second written opinion, should this be explicitly requested.

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Re: Owner: NATIONAL RESEARCH COUNCIL OF CANADA; INSTITUTE FOR MAGNETIC RESONANCE RESEARCH; THE UNIVERSITY OF SYDNEY; MOUNTFORD, Carolyn E.; SORRELL, Tania; HIMMELREICH, Uwe; BOURNE, Roger; and SOMORJAI, Rajmund L.
Patent Application under the Patent Cooperation Treaty (PCT)
Serial No. : PCT/CA02/00216 Filing Date: February 21, 2002
Our Case : P12202
Title : Magnetic Resonance Spectroscopy to Identify and Classify Microorganisms

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Dear Sirs:

COMMUNICATION AND RESPONSE TO 25 OCTOBER 2002 WRITTEN OPINION

This Communication is submitted in response to the Written Opinion issued in connection with the above-identified application on 25 October 2002. In accordance with the 25 October 2002 Written Opinion, a response is due on 25 November 2002.

REQUEST FOR DETAILED SUBSTANTIVE EXAMINATION

The applicant requests detailed substantive examination of the above-noted application.

If following a review of the enclosed amendments and/or arguments, the Examiner is of the view that any of the claims do not possess the requisite elements of novelty, inventive or industrial applicability so as to warrant the granting of patent protection, the Examiner is requested to draw-up a second Written Opinion in respect of this application

AMENDMENTS UNDER ARTICLE 34

In response to the 25 October 2002 Written Opinion, please amend the subject application as follows:

IN THE CLAIMS:

Please replace original pages 63-69 with replacement pages 63-69 attached hereto as Exhibit A. Claims 1, 2, 19, 20, 37, 38 and 40 are being amended.

REMARKS

Claims 1-54 are pending in the present application.

Applicants have submitted replacement pages 63-69. By these submitted pages, claims 1, 2, 19, 20, 37, 38 and 40 have been amended and the remaining claims remain unchanged. After entry of the substituted sheets 63-69, claims 1-54 will be pending and under examination.

In the Written Opinion, the Examiner said that in light of the documents cited in the International Search Report, it is considered that the invention as defined in at least some of the claims does not appear to meet the criteria mentioned in Article 3(1) PCT, i.e. does not appear to be novel and/or to involve an inventive step, referring to the International Search Report, in particular the documents cited X and corresponding claims references.

The only reference cited as a category X reference was J.C. Wallace et al., "Classification of ^{1}H NMR Spectra of Biopsies from Untreated and Recurrent Ovarian Cancer Using Linear Discriminant Analysis", Magn. Res. Med., 38,569-576 (1997) ("Wallace").

Independent claims 1, 19, and 37 have been amended, as well as claims 2, 20, 38 and 40. In claim 1, the end of the preamble has been amended to include the characterizing clause. In clause (c), the step of repeating the cross-validating step has been added, which repeating step was previously recited in claim 2. Support for this amendment may be found in claim 2 as filed.

Similar amendments have been made to claims 19, 20, 37 and 38.

These amendments more clearly distinguish over the Wallace Reference cited in the Search Report. The Wallace reference reports on earlier work of co-inventors herein, and applicants are familiar with this work.

Firstly, the Wallace reference relates to classifying ovarian tissue. The presently claimed invention is directed to classifying microorganisms of unknown species, which is not disclosed in Wallace, and one skilled in the art would not be motivated by Wallace or otherwise to classify microorganisms.

Further, the Wallace reference does not disclose claim 1, clause (b), which recites cross-validating the spectra by selecting a first portion of the spectra from each species, and repeating the cross-validation of step (b) a plurality of times.

For these reasons, Wallace fails to disclose claim 1. For at least the same reasons, Wallace also fails to disclose claims 19 and 37.

In view of the foregoing, applicant respectfully requests that the Examiner reconsider and withdraw the objection set forth in the 25 October 2002 Written Opinion and earnestly solicits a favorable report of the claims being examined.

If a telephone interview would be of assistance in advancing the prosecution of the subject application, applicant's undersigned attorney invites the Examiner to telephone him at the number provided below.

Respectfully submitted,

NATIONAL RESEARCH COUNCIL OF CANADA; INSTITUTE FOR MAGNETIC RESONANCE RESEARCH; THE UNIVERSITY OF SYDNEY; MOUNTFORD, Carolyn E.; SORRELL, Tania; HIMMELREICH, Uwe; BOURNE, Roger; and SOMORJAI, Rajmund L.

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Enclosures (by fax and mail)
Substitute Claims Pages 63 to 69
(containing claims 1 to 54)

We claim:

1. A method for obtaining a statistical classifier for classifying microorganisms of unknown species into known species, characterized by:

   (a) obtaining a plurality of magnetic resonance spectra of each of a plurality of different species of microorganisms whose species is known;

   (b) locating a plurality of maximally discriminatory subregions in the magnetic resonance spectra obtained;

   (c) cross-validating the spectra by selecting a first portion of the spectra from each species, developing linear discriminant analysis classifiers from the first portion of the spectra from each species, and validating the remainder of the spectra from each species using the classifiers from the first portion of the spectra from each species; and

   (d) repeating step (c) a plurality of times, each time selecting as the first portion of the spectra a different portion of the spectra from the species, to obtain a different set of optimized linear discriminant analysis coefficients and classifier spectra for each of the known species of microorganisms, which coefficients and classifier spectra can be used to determine the species of microorganisms whose species are unknown.

2. The method of claim 1, further comprising the step of obtaining a weighted average of the linear discriminant analysis coefficients to obtain final classifier spectra.

3. The method of claim 1, wherein the step of cross-validating the spectra comprises cross validating the spectra by randomly selecting about half of the spectra.

4. The method of claim 2, wherein the step of repeating step (c) a plurality of times comprises repeating step (c) about 1000 times.

5. The method of claim 1, further including the steps of obtaining a plurality of classifier spectra independently, and aggregating the results of the independent classifiers to obtain a consensus diagnosis.

6. The method of claim 1, wherein the microorganisms include bacteria.

7. The method of claim 6, wherein the bacteria includes the species of *Staphylococcus aureus* and *Staphylococcus epidermidis*.

8. The method of claim 6, wherein the bacteria includes the species of *Enterococcus faecalis*, *Enterococcus casseliflavus* and *Enterococcus gallinarum*.

9. The method of claim 6, wherein the bacteria includes the species of *Streptococcus pneumoniae*, *Streptococcus pyogeries* and *Streptococcus agalachae*.

10. The method of claim 1, wherein the microorganisms include fungi.

11. The method of claim 10, wherein the fungi includes pathogenic yeasts.

12. The method of claim 11, wherein the pathogenic yeasts include *Candida albicans*, *Candida parapsilosis*, *Candida tropicalis*, *Candida krusei*, and *Candida glabrata*.

13. The method of claim 11, wherein the pathogenic yeasts include *Cryptococcus* varieties.

14. The method of claim 13, wherein the *Cryptococcus* varieties include *neoformans* and *gattli*.

15. The method of claim 1, wherein the plurality of magnetic resonance spectra of each different species is at least 10.

16. The method of claim 1, wherein the plurality of magnetic resonance spectra of each different species is at least 30.

17. The method of claim 1, wherein the microorganisms include cultured bacterial infections.

18. The method of claim 1, wherein the microorganisms include specimens from a mammal containing bacterial infections.

19. A method for determining the species of a microorganism of unknown species, characterized by:

obtaining magnetic resonance spectra of the microorganism of unknown species, and comparing the spectra obtained with a species classifier, said classifier having been obtained by

(a) obtaining a plurality of magnetic resonance spectra of each of a plurality of different species of microorganisms whose species is known;

(b) locating a plurality of maximally discriminatory subregions in the magnetic resonance spectra obtained;

(c) cross-validating the spectra by selecting a first portion of the spectra from each species, developing linear discriminant analysis classifiers from the first portion of the spectra from each species, and validating the remainder of the spectra from each species using the classifiers from the first portion of the spectra from each species; and

(d) repeating step (c) a plurality of times, each time selecting as the first portion of the spectra a different portion of the spectra from the species, to obtain a different set of optimized linear

discriminant analysis coefficients and classifier spectra for each of the known species of microorganisms; and

selecting, as the species of the unknown species of microorganism, the microorganism whose spectra has the closest match to the spectra of the unknown microorganism species.

20. The method of claim 19, wherein the steps of obtaining the classifier further comprises the step of obtaining a weighted average of the linear discriminant analysis coefficients to obtain final classifier spectra.

21. The method of claim 19, wherein the step of cross-validating the spectra comprises cross validating the spectra by randomly selecting about half of the spectra.

22. The method of claim 20, wherein the step of repeating step (c) a plurality of times comprises repeating step (c) about 1000 times.

23. The method of claim 19, further including the steps of obtaining a plurality of classifier spectra independently, and aggregating the results of the independent classifiers to obtain a consensus diagnosis.

24. The method of claim 19, wherein the microorganisms include bacteria.

25. The method of claim 24, wherein the bacteria includes the species of *Staphylococcus aureus* and *Staphylococcus epidermidis*.

26. The method of claim 24, wherein the bacteria includes the species of *Enterococcus faecalis*, *Enterococcus casseliflavus* and *Enterococcus gallinarum*.

27. The method of claim 6, wherein the bacteria includes the species of *Streptococcus pneumoniae*, *Streptococcus pyogeries* and *Streptococcus agalachae*.

28. The method of claim 1, wherein the microorganisms include fungi.

29. The method of claim 28, wherein the fungi includes pathogenic yeasts.

30. The method of claim 29, wherein the pathogenic yeasts include *Candida albicans*, *Candida parapsilosis*, *Candida tropicalis*, *Candida krusei*, and *Candida glabrata*.

31. The method of claim 29, wherein the pathogenic yeasts include *Cryptococcus* varieties.

32. The method of claim 31, wherein the *Cryptococcus* varieties include *neoformans* and *gattli*.

33. The method of claim 19, wherein the plurality of magnetic resonance spectra of each different species is at least 10.

34. The method of claim 19, wherein the plurality of magnetic resonance spectra of each different species is at least 30.

35. The method of claim 19, wherein the microorganisms include cultured bacterial infections.

36. The method of claim 19, wherein the microorganisms include specimens from a mammal containing bacterial infections.

37. A statistical classifier for classifying microorganisms of unknown species into known species, characterized by:

(a) a spectrometer for obtaining a plurality of magnetic resonance spectra of each of a plurality of different species of microorganisms whose species is known;

(b) a locator for locating a plurality of maximally discriminatory subregions in the magnetic resonance spectra obtained; and

(c) a cross-validator for cross-validating the spectra by selecting a first portion of the spectra from each species, developing linear discriminant analysis classifiers from the first portion of the spectra from each species, validating the remainder of the spectra from each species using the

classifiers from the first portion of the spectra from each species, and repeating the validating step a plurality of times, each time selecting as the first portion of the spectra a different portion of the spectra from the species, to obtain optimized linear discriminant analysis coefficients and classifier spectra for each of the known species of microorganisms, which coefficients and classifier spectra can be used to determine the species of microorganisms whose species are unknown.

38. The classifier of claim 37, wherein the cross-validator obtains a weighted average of the linear discriminant analysis coefficients to obtain final classifier spectra.

39. The classifier of claim 37, wherein the cross-validator cross validates the spectra by randomly selecting about half of the spectra.

40. The classifier of claim 38, wherein the classifier repeats the validating step about 1000 times.

41. The classifier of claim 37, wherein the classifier obtains a plurality of classifier spectra independently, and aggregates the results of the independent classifiers to obtain a consensus diagnosis.

42. The classifier of claim 37, wherein the microorganisms includes bacteria.

43. The classifier of claim 42, wherein the bacteria includes the species of *Staphylococcus aureus* and *Staphylococcus epidermidis*.

44. The classifier of claim 42, wherein the bacteria includes the species of *Enterococcus faecalis*, *Enterococcus casseliflavus* and *Enterococcus gallinarum*.

45. The classifier of claim 42, wherein the bacteria includes the species of *Streptococcus pneumoniae*, *Streptococcus pyogeries* and *Streptococcus agalachae*.

46. The classifier of claim 37, wherein the microorganisms include fungi.

47. The classifier of claim 46, wherein the fungi includes pathogenic yeasts.

48. The classifier of claim 47, wherein the pathogenic yeasts include *Candida albicans*, *Candida parapsilosis*, *Candida tropicalis*, *Candida krusei*, and *Candida glabrata*.

49. The classifier of claim 47, wherein the pathogenic yeasts include *Cryptococcus* varieties.

50. The classifier of claim 49, wherein the *Cryptococcus* varieties include *neoformans* and *gattli*.

51. The classifier of claim 37, wherein the plurality of magnetic resonance spectra of each different species is at least 10.

52. The classifier of claim 37, wherein the plurality of magnetic resonance spectra of each different species is at least 30.

53. The classifier of claim 1, wherein the microorganisms include cultured bacterial infections.

54. The classifier of claim 1, wherein the microorganisms include specimens from a mammal containing bacterial infections.

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Dear Sirs:

Submitted herewith by registered airmail is the original copy of our letter of November 22, 2002 which was forwarded to the International Preliminary Examination Authority by facsimile on November 22, 2002.

Respectfully submitted,

NATIONAL RESEARCH COUNCIL OF CANADA; INSTITUTE FOR MAGNETIC RESONANCE RESEARCH; THE UNIVERSITY OF SYDNEY; MOUNTFORD, Carolyn E.; SORRELL, Tania; HIMMELREICH, Uwe; BOURNE, Roger; and SOMORJAI, Rajmund L.

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